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Effects of Hypothalamic Lesions on the Erythropoietic Response to Hypoxia in Rabbits[1]

By

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Abstract

HALVORSEN, S. *Effects of hypothalamic lesions on the erythropoietic response to hypoxia in rabbits.* Acta physiol. scand. 1964. *61.* 1—19. — Previous studies have indicated that electrical stimulation of the hypothalamus increases erythropoiesis (Seip *et al.* 1961) and erythropoietin production (Halvorsen 1961). The effects of hypothalamic lesions on the erythropoietic response to hypoxia have been investigated in the present study. The lesioned rabbits were exposed to low oxygen and the erythropoietic response evaluated by means of reticulocyte counts. Eleven out of 26 rabbits showed a normal response to hypoxia while 15 showed a reduced response. Of these 15 rabbits, 6 showed a normal response when ACTH was given prior to the hypoxic test, indicating that the reduced response to hypoxia in these rabbits was due to ACTH deficiency following the hypothalamic lesion. The lesions in these 6 rabbits were in the ventral and median part of hypothalamus. Seven out of the 15 rabbits neither responded normally to hypoxia alone nor to hypoxia following ACTH injections. The lesions in these 7 rabbits were in the posterior part of the hypothalamus. It is concluded that an extra pituitary influence on erythropoiesis may be exerted from this region.

Previous studies in this laboratory have indicated that the hypothalamus participates in the regulation of erythropoiesis in rabbits. Electrical stimulation of the hypothalamus increased the reticulocyte level and red cell mass in some of the animals (Seip *et al.* 1961). Plasma from the rabbits responding to hypothalamic stimulation with a reticulocytosis and an elevation of red cell mass increased erythrocyte uptake of Fe^{59} and reticulocytes when injected into starved

that the hypothalamus participates in the regulation of erythropoietin produc *tion and erythropoiesis and that this regulatory mechanism is not mediated* through ACTH alone

The working hypothesis for our studies on the hypothalamic regulation of erythropoiesis has been that the oxygen tension is registered in the hypothalamic area and that the erythropoietins are produced in one or several target organs stimulated via hypothalamus by humoral or nervous routes Although hypo thalamic stimulation increased erythropoiesis ablation studies are necessary to get conclusive evidence for or against the hypothesis of specific regulation of erythropoiesis by the hypothalamus

The effect of hypothalamic lesions on pituitary function has been reviewed several times recently (Harris 1960 Ganong and Forsham 1960 Fortier 1962) Although there are some differences of opinion on the localization of the hypo thalamic areas regulating pituitary hormone secretion there is general agree ment that the output of adrenocorticotrophic (ACTH) thyrotrophic (TSH) and gonadotrophic hormones is regulated via hypothalamus Recently Reichlin (1961) and Franz *et al* (1962) have provided evidence that the hypothalamus also regulates growth hormone (GH) secretion

Several studies have documented the influence of pituitary hormones on erythropoiesis both in hypophysectomized and in intact animals (van Dyke *et al* 1954 Evans *et al* 1961 Halvorsen 1963) The hormonal control of erythro poiesis has been reviewed by Gordon (1959), Linman and Bethell (1960) and Remmele (1963) There is general agreement that ACTH and corticosteroids increase erythropoiesis both in intact and hypophysectomized animals Testos terone and thyroxin also stimulate erythropoiesis and most authors believe that the effect of these hormones on erythropoiesis is due to their calorigenic effect (Evans *et al* 1961) The effects of GH on erythropoiesis are more complex The hormone causes a hyperplasia of the erythroid elements of the bone marrow and a peripheral reticulocytosis when given to hypophysectomized rats (Meineke and Crafts 1956) Remmele (1963) states that GH has an effect directly in the bone marrow

Previous studies on the participation of the central nervous system in regula tion of erythropoiesis have been reviewed by Seip (1953) Komiya (1956) Seip *et al (1961) and Remmele (1963) The effects of hypothalamic lesions on* erythropoiesis have previously been studied by Komiya and Hayashida (cit Komiya 1956) who found increases in hemoglobin and erythrocytes following lesions in the tuber cinereum Linke (1958 1959) has produced hypothalamic lesions in rabbits and exposed the operated animals to hypoxia In some of these rabbits the reticulocyte response to hypoxia was reduced but the possibility of pituitary deficiency in the operated animals was not discussed Piliero *et al* (1962) investigated the erythropoietic response to long term hypoxia in rats with hypothalamic lesions and found no difference between the operated and sham-operated animals

Little is known about the role of the autonomic nervous system in erythropoiesis. There is some direct evidence of specific participation. Reticulocyte release has been found following injections of cholinergic drugs (Seip 1953). Experiments by Somogyi (1938) on the effect of extirpation of cervical sympathetic ganglia and by Komiya (1956) and Takaku *et al.* (1962) on the effect of splanchnic nerve resection all indicate that the autonomic nervous system is an important link in the regulation of erythropoietic responses to hypoxia, at least in acute experiments. As the hypothalamic area plays an important role in the integration of the autonomic nervous system (Ingram 1958), lesioning procedures may influence functions which secondarily may affect erythropoiesis.

Several previous studies indicate that the hypothalamus exerts a specific influence on erythropoiesis. The studies by Seip *et al.* (1961) provide the most direct evidence in favor of this assumption. None of these studies have, however, been considered conclusive and it was the purpose of the present investigation to study this possibility further in ablation experiments. The crucial problem is to separate the role of the hypothalamus and the role of the pituitary gland and the report deals with these two questions. Do hypothalamic lesions influence the erythropoietic response to hypoxia? If the erythropoietic response to hypoxia is reduced following hypothalamic lesions, do pituitary hormones normalize the response?

Material and methods

Hypothalamic lesions have been produced electrolytically in 46 adult male rabbits. Twenty of these died during the operation or postoperatively and the material consists of the 26 surviving rabbits. During the first part of the study the rabbits were anesthetized with Chloralose Urethane i. v. and Urethane i. p. while the last 8 rabbits received i. v. Nembutal. In a few rabbits ACTH, normal saline and 6 per cent glucose were given postoperatively. All the rabbits were placed in a temperature regulated chamber (26° C) following the operation.

During the operation the head of the rabbits was immobilized in a standard Horsley Clarke carriage with the special head holder for rabbits described by Sawyer *et al.* (1954). A monopolar electrode 0.3 mm thick and insulated except for 1.5 mm at its tip, was inserted through a small burr hole in the skull and oriented by the use of the stereotaxic coordinates described by the same authors. Bilateral lesions were attempted in all experiments by insertion of the electrodes 1 mm from the midline on both sides. In the first 33 rabbits one lesion only was made on each side while in the last 13 animals two points 2 mm apart were electrolyzed on each side in order to achieve a more extensive destruction. A direct current of about 5 mA was passed through the inserted electrode for 35 to 60 sec., the indifferent electrode being placed on the edge of the cut skin.

At the termination of the experiments the animals were killed, the brain removed and the whole brain was fixed in 10 per cent formalin alcohol for at least 7 days, after which a block of tissue containing the hypothalamus was cut out, embedded in paraffin, serially sectioned in a frontal plane at 20 μ and every twentieth section mounted and stained with thionine. Later, the number of sections was supplemented so that at least every tenth section was examined in the region of the lesion. The location of the lesions

that the hypothalamus participates in the regulation of erythropoietin production and erythropoiesis, and that this regulatory mechanism is not mediated through ACTH alone

The working hypothesis for our studies on the hypothalamic regulation of erythropoiesis has been that the oxygen tension is registered in the hypothalamic area and that the erythropoietins are produced in one or several target organs stimulated via hypothalamus by humoral or nervous routes Although hypothalamic stimulation increased erythropoiesis, ablation studies are necessary to get conclusive evidence for or against the hypothesis of specific regulation of erythropoiesis by the hypothalamus

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Table I shows the reticulocyte response to hypoxia in intact rabbits. In this and the following tables the reticulocyte values tabulated are the mean of two counts before (Initial) and the mean of the two highest counts between the second and the sixth day after the hypoxic test (Highest). The initial reticulocyte values marked ( ) are based on one count only. The third column of reticulocyte values represent the difference between the mean of the two highest and the mean of the initial counts.

Reticulocyte response to hypoxia in intact rabbits.

Rabbit no	Hours of hypoxia	Reticulocytes per cent Initial	Highest	Difference
C 1	8	1.95	4.60	2.65
C 2	8	1.80	3.95	2.15
C 3	8	1.35	3.78	2.43
C 4	8	2.35	4.85	2.50
C 5	8	1.18	3.73	2.55
C 6	4	1.98	4.85	2.87
C 7	5	1.48	2.88	1.40
C 8	5	1.23	3.55	2.32
	Amount of bleeding ml/kg			
C 9	15	3.00	5.37	2.37
C 10	20	2.55	5.57	3.02

Results

The mean reticulocyte curve in 5 intact rabbits following hypoxia of the described degree and duration is shown in Fig. 5 (dotted lines). The variations in reticulocyte response from animal to animal were small. This was especially true when the difference between the mean of the two highest values 2 to 6 days after the hypoxia is compared with the mean of two reticulocyte values before hypoxia (Table I). The mean of these differences is 2.46 per cent with a standard deviation of 0.19 per cent. Table I also shows the reticulocyte response to 4—5 hours of hypoxia in three rabbits and to bleeding (15 and 20 ml/kg b. w.) in two rabbits.

The reticulocyte response to hypoxia in the 5 intact rabbits exposed to 8 hours of hypoxia has been considered to be the *normal* response with the described degree and duration of hypoxia and in rabbits with *normal* hemoglobin levels and a steady state of erythropoiesis before the hypoxic test.

Based on the results in intact rabbits, reticulocyte increases smaller than 1.7 per cent (mean — 4 standard deviations) measured as described above between the second and sixth day after hypoxia have been considered as reduced responses, increases above 3.2 per cent (mean + 4 standard deviations) as high responses and increases between 1.7 and 3.2 per cent as normal responses.

Table II shows the main experimental data in the group of lesioned rabbits with a normal or high reticulocyte increase following hypoxia The time of the hypoxic test after the lesion and the hemoglobin level at the time of the test are tabulated Rabbit no 15 and 38 also responded to anemic hypoxia with a reticulocyte increase

Group I Normal reticulocyte response to hypoxia

Rabbit no	Time of hypoxia after lesion (days)	Hb (g)	Reticulocytes per cent Initial	Highest	Difference
4	7	10.2	2 30	4 05	1 75
7	9	15 4	1 43	3 18	1 75
8	8	13 8	1 18	4 90	3 72
9	5	9 6	0 50	3 53	3 03
11	2	12.5	2 13	6 75	4 62
15	16	14 3	1 80	3 50	1 70
25	16	13 4	1 40	3 30	1 90
38	10	12 5	2 60	4 30	1 70
39	9	12 3	3.28	6 52	3.24
43	9	11 4	1 25	5 13	3 88
44	11	11 6	0 88	3 75	2 87

Of the 26 rabbits which survived and were subjected to hypoxia, 11 responded with a normal or high reticulocyte response to the hypoxic test while 15 exhibited a reduced response Two of these 15 animals died before further studies could be performed whereas the remaining 13 rabbits were given ACTH prior to a repeated hypoxic test Six of these 13 rabbits responded with a normal or high reticulocyte response to the repeated test Thus the material may be divided into four groups

Group I	Normal reticulocyte response to hypoxia	11
Group II	Reduced reticulocyte response to hypoxia normal response following pre treatment with ACTH	6
Group III	Reduced reticulocyte response to hypoxia with and without pre treatment with ACTH	7
Group IV	Reduced reticulocyte response to hypoxia Incomplete studies	2

The data from *Group I* are shown in Table II which as the following tables gives the interval between the hypoxic test and the operation the hemoglobin at the time of the test the means of two reticulocyte values before hypoxia the means of the two highest reticulocyte values after hypoxia and the differences between these two means The localization of the hypothalamic lesions is shown in Fig 1

The data from *Group II* are shown in Table III In some of the rabbits in this group the reticulocyte response following ACTH and hypoxia was markedly increased One rabbit 14) was given ACTH without subsequent hypoxia and

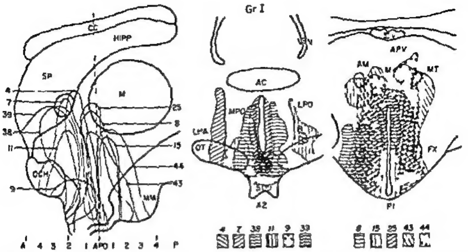

Fig 1 Localization of the hypothalamic lesions in the rabbits with a normal or high reticulocyte response to hypoxia (Group I) projected to the midsagittal plane and in transverse sections through the planes A 2 and P 1

Table III shows the data for the rabbits responding to hypoxia following pre treatment with ACTH

Group II Reduced reticulocyte response to hypoxia Normal reticulocyte response to hypoxia following pre treatment with ACTH

Rabbit no	Time of hypoxia after lesion (days)	Hb (g)	Hormone therapy	Reticulocytes per cent Initial	Highest	Difference
10	4	11.4	None	0.53	1.68	1.15
	14	10.5	None	1.05	1.80	0.75
	28	10.5	ACTH	2.15	4.15	2.00
	41	7.6	Cortisone	2.50	5.23	2.73
14	14	12.6	None	0.50	1.53	1.03
	21	13.0	ACTH	1.77	7.30	5.53
20	9	11.4	None	1.65	2.35	0.73
	19	10.4	ACTH	2.70	6.05	3.35
24	9	13.0	None	1.10	2.35	1.25
	25	14.6	ACTH	1.80	4.63	2.83
3[illegible]	[illegible]3	13.4	None	1.93	3.03	1.15
	35	12.5	ACTH	1.60	5.10	3.50
	41		Cortisone	1.63	4.30	[illegible]67
33	27	14.6	None	3 [illegible]0	4.95	1.25
	44	14.6	ACTH	2.15	4.13	1.93

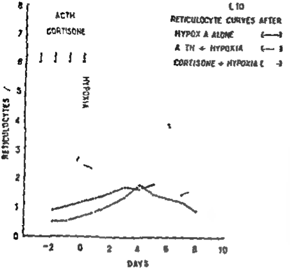

Fig 2 The reticulocyte curves following hypoxia alone and hypoxia after pre treatment with ACTH and Cortisone in rabbit No 10

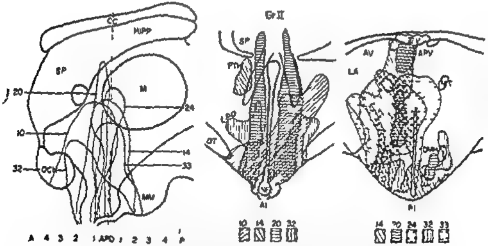

Fig 3 Localization of the hypothalamic lesions in the rabbits with a reduced response to hypoxia alone and a normal response following pre treatment with ACTH (Group II) projected to the midsagittal plane and in transverse sections through the planes A 1 and P 1

there was no increase in reticulocytes Two rabbits (10 32) received cortisone prior to hypoxia and this had the same effect as ACTH in normalizing the reticulocyte response to hypoxia (Fig 2 10) The lesions in this group are shown in Fig 3

The data from *Group III* are presented in Table IV and the hypothalamic lesions in Fig 4 This group is divided into subgroups in accordance with the results of the further studies

Table IV shows the data in the group of rabbits which did not respond to hypoxia with and without pre treatment with ACTH

Group III Reduced reticulocyte response to hypoxia alone and to hypoxia following ACTH

III a. Normal reticulocyte response to hypoxia following ACTH plus GH

Rabbit no	Time of hypoxia after lesion (days)	Hb (g)	Hormone therapy	Reticulocytes per cent Initial	Highest	Difference
22	9	16 3	ACTH	2 28	3 53	1 25
	42	14 6	ACTH+GH	2.95	7.23	4.28
	54	12 3	GH	1 48	4 05	2 57
	90	16 3	None	2 45	4 00	1.55
46	5		None	1 00*	0 88	−0 12
	14		ACTH	1 08	2.35	1.27
	23	12 5	ACTH+GH	1 58	3 55	1 97

III b Normal reticulocyte response to anemic hypoxia

Rabbit no	Time of hypoxia after lesion (days)	Amount of bleeding (ml/kg)	Hb (g)	Hormone therapy	Reticulocytes per cent Initial	Highest	Difference
31	7		12 8	None	1 35	2 15	0 80
	45			ACTH	2.08	2 93	0 85
	59	17	16 8 10.5	None	1 15	4 70	3 55
34	18		12 8	None	1 45	1 75	0 30
	28		13 4	ACTH	1.93	3 25	1 32
	41		11 3	ACTH+GH	2 13	2 58	0 45
	54		14 3	ACTH+Test	3 03	4 03	1 00
	66			ACTH+GH+TSH+Test	3 00	3 68	0 68
	75	20	13 0 10 5	None	0 50*	2 87	2.37

a) Two of the rabbits (22 46) responded to hypoxia when ACTH and GH were given prior to the hypoxic tests In rabbit No 46 ACTH and GH without hypoxia failed to increase the reticulocytes while in No 22 ACTH and GH combined and GH alone increased the reticulocyte level on the first day after the last injection but with a fall to previous levels on the following days According to the description of the normal reticulocyte response to hypoxia this short term effect is not transferred through erythropoietin

Table IV (cont.)

Group III. Reduced reticulocyte response to hypoxia alone and to hypoxia following ACTH

III c. Reduced reticulocyte response to the hypoxic tests

Rabbit no.	Time of hypoxia after lesion (days)	Amount of bleeding (ml/kg)	Hb (g)	Hormone therapy	Reticulocytes per cent Initial	Highest	Difference
30	5		10.3	None	1.45	1.55	0.10
	33		15.6	ACTH	1.05	2.70	1.65
	52		16.1	Test	0.88	2.38	1.50
	60		16.8	ACTH+GH+TSH+Test	0.78	2.08	1.30
	83	16	17.0 9.8	None	0.70	0.70	0.00

III d. Incompleted studies

Rabbit no.	Time of hypoxia after lesion (days)	Hb (g)	Hormone therapy	Reticulocytes per cent Initial	Highest	Difference
26	7	11.6	None	3.10	4.35	1.35
	32	12.5	None	2.35	1.75	−0.60
	42	14.0	ACTH	0.78	2.13	1.35
41	6	12.8	None	1.25	1.90	0.65
	16	11.6	ACTH	0.68	1.53	0.85

b) Two rabbits (31, 34) failed to respond to hypoxia following administration of hormone, but had a reticulocyte increase following anemic hypoxia.

c) One rabbit (30) failed to respond normally either to hypoxic hypoxia with hormone pre-treatment or to anemic hypoxia. The hemoglobin in this rabbit increased from 10.3 to 17.0 g. The reticulocytes were rather low throughout the observation period. The weight remained constant. The RCM was not determined in this rabbit.

d) Two rabbits (26, 41) were subjected only to hypoxic tests with and without ACTH pre-treatment and died before further tests could be done.

The data from *Group II* are shown in Table V. In these two rabbits (13, 42) only one hypoxic test without hormone pretreatment was performed.

Although the reticulocyte counts in many rabbits were low following the hypothalamic lesions, only one rabbit (42) showed a definitely different pattern

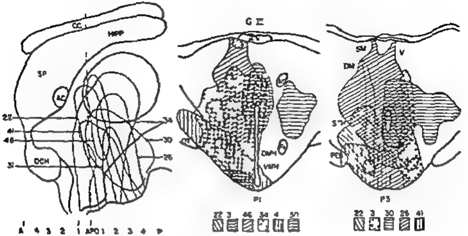

Fig. 4. Localization of the hypothalamic lesions in the rabbits with a reduced response to hypoxia with and without ACTH pre-treatment (Group III) projected to the midsagittal plane and through the planes P 1 and P 3.

from the others. Seven to ten days after the hypothalamic lesion the reticulocyte count increased markedly (max. 10.6 per cent) and was higher than usual thereafter. The hemoglobin and erythrocytes remained constant. In this rabbit a red cell mass determination was performed to see whether any polycythemia had developed, but the red cell mass was 16.3 ml/kg b.w., which is slightly below the mean previously found in rabbits. In this rabbit there was a large central lesion in the preoptic area.

Most of the rabbits lost weight during the first week after operation, but the weight loss was usually slight and of short duration. Of the rabbits alive more than 14 days after the operation, 3 (8, 15, 39) lost weight markedly, about 50 g/day, and developed a condition similar to hypothalamic cachexia (Szentogothai *et al.* 1962). All three responded with a high or normal response to hypoxia alone. Many rabbits had increased appetites and a few became obese. Four rabbits (13, 26, 38, 42) put on more than 15 g/day. One of these (38) had

Table V shows the data in two rabbits which died before further studies could be performed.

Group IV. Incompleted studies

Rabbit no.	Time of hypoxia after lesion (days)	Hb (g)	Hormone therapy	Reticulocytes per cent Initial	Highest	Difference
13	13	12.3	None	2.80	3.45	0.65
42	33	12.3	None	3.[illegible]5	5.15	1.40

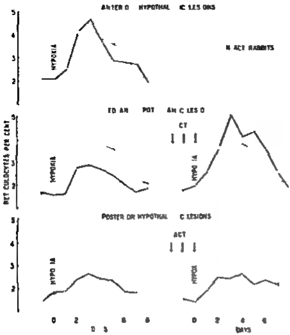

Fig 5 Mean reticulocyte responses to hypoxia in lesioned rabbits grouped according to the main localization of their lesions (solid lines) compared with the mean response to hypoxia in five intact rabbits (dotted lines) The effect of ACTH prior to repeated hypoxic tests is also shown Anterior hypothalamic lesions Rabbit No 4 7 9 10 11 38 39 4? Median hypothalamic lesions Rabbit No 8 14 20 24 25 32 33 46 Posterior hypothalamic lesions Rabbit No 13 15 22 26 30 31 34 41 43 44

a low normal response to hypoxia alone the other had reduced responses Four rabbits (10 20 24 33) put on between 10 and 15 g/day They all failed to respond to hypoxia alone but responded after pre treatment with ACTH

Correlation with anatomical data

Most of the operated animals had relatively large lesions in the thalamic and hypothalamic regions The lesions cover most parts of the hypothalamus except the lateral hypothalamic areas which have not been symmetrically lesioned although some animals had unilateral lesions in these areas

The rabbits in *Group I* had lesions both in the preoptic area the ventral thalamus the supraoptic and infundibular regions and in the mammillary areas (Fig 1) The lesions in the preoptic and supraoptic regions were relatively large and symmetrical Symmetrical lesions were also produced in the walls of the third ventricle in the middle infundibular region and in 2 rabbits in the premammillary and mammillary areas as well Only one of the lesions in this group extended down to the infundibulum

The lesions in *Group II* all involved the middle supraoptic and infundibular regions and extended downwards to the floor of the third ventricle although a direct connection could not be ascertained in all cases (Fig 3) The lesions could not be separated entirely from some of those in Group I

The lesions in *Group III* are shown in Fig 4 All the lesions in this group destroyed the posterior part of the hypothalamus and most of them affected the

premammillary and mammillary regions. Although there was some overlap with the lesions in Group I the localization within the group was fairly uniform.

The lesions in the two rabbits of *Group* II are not illustrated. Rabbit no. 42 had a large symmetrical lesion in the preoptic area, and no. 13 a lesion in the ventral infundibular and premammillary regions.

The lesioned rabbits have also been grouped according to the localization of the lesions. Fig. 5 shows the means of the reticulocyte responses to hypoxia in rabbits with lesions mainly in the anterior, median and posterior hypothalamus (solid lines) compared with the mean response to hypoxia in 5 intact rabbits (dotted lines). The rabbits which did not respond normally to hypoxia alone have been given ACTH prior to a repeated hypoxic test, and Fig. 5 also shows the means of these responses. Anterior hypothalamic lesions have no influence on the reticulocyte response to hypoxia. Median and ventral hypothalamic lesions reduce this response and the response is normalized when ACTH is given prior to the hypoxic test. Posterior hypothalamic lesions reduce the reticulocyte response to hypoxia both without and with ACTH pretreatment.

The lesions in the rabbits showing the most marked weight changes were scattered throughout the hypothalamus but common to all were a median localization in the structures close to the third ventricle. The number of observations was too small to differentiate between those with weight loss and those with weight increase.

Comments

Reticulocyte counts have been used as parameter to test the erythropoietic responses in the present study. Seip (1953) found reticulocyte counts to be a reliable index of erythropoiesis in man when an accurate counting method was used, and in previous studies we have found reticulocyte counts reliable also in rabbits (Seip *et al.* 1961, Halvorsen 1963). Two exceptions must, however, be considered. In conditions with great changes in the number of circulating erythrocytes the reticulocyte percentages must be corrected for the changes in number of erythrocytes or the changes in red cell mass. The reticulocyte may also appear in the peripheral blood at different stages of maturation and such changes may influence the counts. The time of the reticulocyte peak following a stimulus is important in this connection. The effect of reticulocyte release will appear within a few hours, while effects due to increased erythropoiesis have a maximal reticulocyte peak 3 to 4 days after the hypoxic stimulus or erythropoietin injection. From own studies of the reticulocyte curves following hypoxia in intact rabbits and from studies of the reticulocyte curves following erythropoietin injection (Gurney *et al.* 1961), one may conclude th[illegible]

within the first day or after the sixth day cannot be considered to be the result of erythropoietin increase following short term hypoxia

When daily reticulocyte counts are performed curves rather than single values may be compared and this gives a more reliable index of erythropoiesis In the presentation of the data in this study it has however been chosen to use the means of two reticulocyte values before and two after the hypoxic test and to express the response as the difference between these two means The actual values can then be given and the responses may be more easily compared

The reticulocyte response to hypoxia was tested in [redacted] intact rabbits In previous studies lower oxygen tensions have been necessary to produce an increase in erythropoiesis in hypophysectomized animals than in intact animals (Feigin and Gordon 1950) and as low as 6 5 — 8 5 per cent oxygen has therefore been used in the present study Four to five and eight hours of hypoxia were tested and it was found that with both periods a definite reticulocyte increase occurred Eight hours hypoxia was chosen because the response to this duration of hypoxia was relatively constant

It may be questioned whether or not a response to hypoxia in experimental animals with possible changes in erythropoiesis shortly before the hypoxic test may be compared with the response in intact animals In a series of 8 rabbits followed for 14 days with 3 to 7 counts in each rabbit the highest difference between single values in one rabbit was 1 76 per cent The mean increase of reticulocytes following hypoxia was 2 46 per cent with a standard deviation of 0 19 per cent Based upon such considerations 1 70 per cent has been chosen as the limit between a normal and a reduced response although this limit cannot be given too much significance in borderline responses

Hypothalamic lesions may change the output of pituitary hormones An increased output may increase both oxygen consumption and because of this the erythropoietic response to hypoxia Reduction of pituitary hormone secretion may however reduce oxygen consumption and the erythropoietic response to hypoxia In order to overcome a possible pituitary hypofunction in the lesioned rabbits pituitary hormones have been given before the hypoxic tests if there was a reduced response to hypoxia alone

This experimental set up is valid only when the pituitary hormones or pituitary extracts do not have any specific effect on erythropoiesis Osnes (1960) and later Shirakura (1961) postulate an effect of ACTH on an erythropoietic principle produced in the kidneys while most other investigators maintain that the effect of ACTH is through cortisone and through the calorigenic action of the latter hormone (Evans *et al* 1961 Remmele 1963) Some observations seem to indicate that GH may have a direct effect on the bone marrow (Meineke and Crafts 1956 Remmele 1963) In the case of GH supplement before a hypoxic test it may be difficult to tell what is due to the hormone and what to hypoxia

The reticulocyte counts following the hypothalamic lesions varied In most rabbits (15 out of 26) the counts were lower than the mean of the 8 intact rabbits previously referred to (1 66 per cent) while in a few rabbits the counts were definitely higher than normal In one rabbit (42) there was a spontaneous rise in reticulocytes This rabbit had a large lesion in the preoptic area Although the reticulocytes were low for a long period in some animals only one developed anemia (no 10 Hb = 7 6 g) The cause of the low reticulocytes and the anemia in this rabbit was most probably a reduced pituitary function because the reticulocytes increased after hormone injections and hypoxia

Eleven of the 26 lesioned rabbits responded to hypoxia with a normal or high reticulocyte increase (Group I) The lesions in these 11 rabbits were mainly in the anterior hypothalamus but two of them also in the premammillary and mammillary areas None of these lesions have reduced the erythropoietic response to hypoxia

Six rabbits had a reduced response to hypoxia alone but responded with a normal or high reticulocyte increase when ACTH was given prior to the hypoxic test (Group II) Although ACTH without hypoxia was only tested in one rabbit it is most likely that the mechanism was the same in all 6 animals i e an increased response to hypoxia and not to ACTH alone Cortisone had the same effect as ACTH in the two rabbits which received both hormones indicating that the effect of ACTH was mediated through the adrenal cortex It must be concluded that the regulatory mechanism of erythropoiesis was intact in these 6 rabbits following the lesioning procedure The hypothalamic lesions in these 6 rabbits were all in the ventral parts of hypothalamus in regions where it is known that lesions may produce reduction in the pituitary hormone secretion (Fortier 1962)

Seven rabbits did neither respond normally to hypoxia alone nor to hypoxia following ACTH treatment In 5 of these rabbits further studies have been performed Although these rabbits differed in response to the further tests they represent a group (Group III) in which hypothalamic lesions have reduced the erythropoietic response to hypoxia and in which ACTH did not influence the response If hypothalamus has an influence on erythropoiesis other than through the hypophysis it probably originates in the areas in which these seven rabbits had their lesions The lesions in all the 7 rabbits were in the posterior part of the hypothalamus and none of them extended down towards the infundibulum

Two of the 7 rabbits responded to hypoxia when GH was given in addition to ACTH before the hypoxic test (Group III a) Although ACTH and GH alone did not increase reticulocytes in one of these rabbits (46) they did in the other (22) and this effect may be more complicated The reticulocytes are not reliable indices in this situation because of the reticulocytosis often seen when GH is given The lesions in these 2 rabbits were in the posterior hypo-

thalamus and they destroyed a common area in the posterior part of nucleus paraventricularis

In 3 rabbits of the 7 which did neither respond to hypoxia alone nor to hypoxia after pre treatment with ACTH, the reticulocyte response to anemic hypoxia was also tested Two of the rabbits (Group III b 31 and 34) had a rise in reticulocytes following bleeding It is not likely that there are differences in the mechanism of response to different types of hypoxia, although such a possibility cannot be excluded Because of reduced responses to several hypoxic tests with and without hormone pretreatment an impaired erythropoiesis regulatory mechanism may however, be suspected in these 2 rabbits One rabbit (Group III c, 30) responded normally neither to hypoxic nor to anemic hypoxia but there was a slight however reduced response after being pretreated with hormones The hemoglobin level increased from 10 3 to 17 0 g an increase which was probably not due to the 4 short hypoxic tests performed As the red cell mass was not determined one does not know whether this represents a real polyglobulia The findings in this rabbit may be related to previous observations of increase in hemoglobin and red cells following hypothalamic lesions The lesion in this rabbit was large and symmetrical in the mammillary areas and extended more posteriorly than in the other rabbits The two rabbits (30 34) that were studied with a combination of several pituitary hormones represent the most direct evidence in this study that the hypothalamus has an influence on erythropoiesis separate from the regulation through the pituitary hormones Whether this influence is a specific influence on erythropoietin production or erythropoiesis or whether it is secondary through changes in circulation or respiration cannot be stated and further studies with lesions in the posterior part of hypothalamus are necessary to elucidate the possibility further One may conclude however that the mechanism of hypothalamic influence on erythropoiesis cannot be explained only on the basis of changes in the secretion of pituitary hormones

The present study confirm Linke s observation that hypothalamic lesions may reduce the reticulocyte response to short term hypoxia It further shows that the reduced erythropoietic response in the lesioned animals may be due to pituitary hypofunction but it also shows that an extra pituitary mechanism may be the cause of the reduced erythropoietic response to hypoxia in rabbits with posterior hypothalamic lesions The discrepancies between the findings in this study and the studies of Piliero *et al* (1962) may be related to the size and localization of the lesions

It is interesting to observe that the 3 rabbits which developed a cachectic condition all responded normally to hypoxia Starvation usually reduces oxygen need and erythropoiesis and it is most likely that the reason for the positive response in these rabbits is connected with the observation reported by Szentogothai *et al* (1962 that the metabolic rate is increased in animals with hypothalamic cachexia

Conclusions

The effect of hypothalamic lesions on the erythropoietic response to hypoxia has been tested in 26 adult male rabbits The lesioned rabbits were exposed to [illegible] 5—8 5 per cent oxygen in nitrogen for 8 hours and the reticulocytes counted daily during the following 6—8 days Reticulocyte increases expressed as the difference between the mean of the two highest counts after hypoxia and the mean of two counts before hypoxia smaller than 1 7 per cent have been considered as reduced responses In rabbits with reduced reticulocyte response to hypoxia alone repeated hypoxic tests were performed following pre treatment of the animals with pituitary hormones

Eleven out of the 26 lesioned rabbits showed a normal or high reticulocyte response to hypoxia (Group I) while the remaining 15 rabbits showed a reduced response Thus the present study confirms Linke s previous observation of reduced reticulocyte response to hypoxia in animals with hypothalamic lesions

The mechanism of the reduced response may apparently differ depending upon the site of the lesion In 6 rabbits in the present study (Group II) the cause of the reduced response was probably decrease in ACTH secretion because ACTH administration before repeated hypoxic tests normalized the response Cortisone had the same effect as ACTH in normalizing the response indicating that the effect of ACTH was mediated through the adrenal cortex. The hypothalamic lesions in these 6 rabbits were in the ventral part of the middle supra optic and infundibular regions

In 7 rabbits (Group III) ACTH given before repeated hypoxic tests did not normalize the reticulocyte response Two of the 7 rabbits responded however normally when ACTH and GH was given before the hypoxic test and two showed a reticulocyte response to bleeding One rabbit responded normally neither to hypoxic nor to anemic hypoxia Although the 7 rabbits in this group differed in response to further hypoxic tests it is concluded that the hypothalamic lesions in these animals have reduced the erythropoietic response to hypoxia through an extra pituitary mechanism The lesions in these rabbits were all localized in the posterior part of hypothalamus

Three rabbits with marked weight loss had a normal or high reticulocyte response to hypoxia probably related to an increased metabolic rate

The correlation between the results of the hypoxic tests and the anatomical data is not complete but it may be concluded generally that lesions in the pre optic area did not reduce the erythropoietic response to hypoxia Lesions in the ventral part of the hypothalamus may reduce the response but this response is often normalized when pituitary hormones are given before the hypoxic tests Lesions in the posterior hypothalamus may also reduce the erythropoietic response and pre treatment with pituitary hormones has less influence in rabbits with localization of the lesions to this region It is concluded that an extra pituitary influence on erythropoiesis may be exerted from this region

The present study elucidates the caution needed in interpreting effects of hypothalamic ablation studies on erythropoiesis without considering the hypothalamic control of the pituitary hormones and their influence on erythropoiesis

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Abbreviations

The abbreviations used in Fig 1 Fig 3 and Fig 4 are mainly according to Sawyer *et al* (1954)

AC — Anterior commissure
AM — N anteromedialis
APV — N paraventricularis anterior
AV — N anteroventralis
CC — Corpus callosum
DM — N medialis dorsalis
DMH — N dorsomed alis hypothalami
FX — Fornix
HIPP — Hippocampus
LA — N lateral s anterior
LHA — Lateral hypothalamic area
LPO — Lateral preoptic area
M — Massa intermedia
MM — N mamm llaris medialis
MPO — Medial preoptic area
MT — Mammillo thalamic tract
NF — Infundibulum
OCH — Optic chiasma
OT — Optic tract
PED — Basis pedunculi
PTH — Prethalamic nuclei
SM — Stria medularis
SOD — N supraopticus diffusus
SP — Septum
VEN — Ventricle
VMH — N ventromedial s hypothalami
III V — Third ventricle

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The Digestion and Absorption of Lactose by the Intact Rat

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Abstract

DAHLQVIST, A. and D. L. THOMSON. *The digestion and absorption of lactose by the intact rat.* Acta physiol. scand. 1964. 61. 20—33. — The digestion and absorption of 2 different doses of lactose was compared with that of equivalent amounts of glucose and galactose. The sugars were administered intragastrically together with a water soluble, non absorbable marker, polyethylene glycol, in order to study the rate of movement of the sugars through the gastro-intestinal tract and to determine their site of absorption. Only the larger dose of lactose showed an effect on the rate of transit of the sugars, and this effect was confined to the distal third of the small intestine. When the monosaccharides were fed, they were almost completely absorbed in the upper two-thirds of the small intestine, glucose being absorbed more rapidly than galactose. Lactose was absorbed in all parts of the small bowel, and after the larger dose, as much as 25 % of the amount of sugar fed was absorbed from the colon. Large amounts of sugar were found in the distal small intestine and colon as long as 6 hours after lactose administration. The caecal contents contain large amounts of lactase, a considerable fraction of which is enzymatically different from small intestinal mucosal lactase. This caecal lactase is probably of bacterial origin. In the small intestine, the rate of hydrolysis of lactose was rate limiting for its absorption. In the large bowel, lactose was hydrolysed more rapidly than the monosaccharides could be absorbed.

Lactose is a disaccharide which has considerable importance in mammalian nutrition, both from the quantitative and qualitative points of view. It is the only carbohydrate present in large amounts in milk, and its administration influences the intestinal flora and the absorption of calcium and related metal ions (Pigman 1957). In large doses it also has a laxative effect. These effects may be related to the locus of absorption of lactose in the gastrointestinal tract, the rate of passage of lactose through the intestine as compared with its corresponding monosaccarides (glucose and galactose), and the length of time that sugar remains in the intestine after lactose feeding.

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In the present investigation we have therefore studied the absorption of lactose in the intact rat and compared it with that of a glucose galactose mixture An unabsorbable reference substance has been used to calculate the rate of passage of the meal and to determine the site of absorption of the sugars The technique is the same as that recently used for studies of the absorption of other disaccharides (Dahlqvist and Thomson 1963 a and b) The rate of hydrolysis of lactose in vivo has been compared with its rate of hydrolysis in vitro by homogenates of the different parts of the gastrointestinal tract

Materials and methods

Animals Male albino rats of the Sprague Dawley strain weighing 193 ± 37 g (mean ± S D) were used *The rats were fasted but allowed water for 16—18 hours before* each experiment

Diet The rats were fed on a commercial pellet diet (diet no 210 Anticimex Stockholm) which according to the manufacturer contained 24 8 % protein and 3 7 % fat On analysis of this diet with the anthrone method (performed as described by Scott and Melvin (1953) but with heating at 100 C for 7 5 min instead of at 90 C for 16 min) we found 51 % carbohydrate using glucose as the standard Of the carbohydrate fraction 86 % was precipitated by 70 % ethanol (polysaccharides) The ethanol soluble (oligosaccharide) fraction on paper chromatography (Whatman paper no 1 run descendent with ethylacetate acetic acid water 9 2 2 for 20 hours) showed two spots one with a mobility similar to that of sucrose the other one similar to lactose Enzymic assay of sucrose with the method described earlier (Dahlqvist and Thomson 1963 a) and of lactose with the method described below showed the oligosaccharide fraction to contain 72 % lactose and 24 % sucrose Thus the diet contains 44 % polysaccharides 5 1 % lactose and 1 7 % sucrose

Sugars Lactose (4 (β D galactopyranosyl) D glucose) monohydrate and glucose were obtained from Baker Chem Co (Phillipsburg N J U S.A) and galactose from Merck A G (Darmstadt W Germany) *All were of analytical grade purity*

On complete hydrolysis 1 0 g of lactose monohydrate will yield 0 5 g of glucose and 0 5 g of galactose

Absorption tests *The technique of the absorption tests was similar to that previously* described for sucrose (Dahlqvist and Thomson 1963 a) The sugar was administered together with 50 mg of polyethylene glycol in 4 ml of water through a stomach tube In one series of experiments 800 mg of sugar (either lactose or an equimolar mixture of glucose and galactose) were given to each rat in another series 400 mg After the time allowed for asorption the animals were killed and the stomach upper middle and lower third of the small intestine and the large intestine homogenized and analysed separately

Analytical methods The determination of polyethylene glycol and the precipitation of protein before sugar analysis were performed as described earlier (Dahlqvist and Thomson 1963 a) The assay of sugars in the protein free filtrates was performed as follows

Determination of free glucose The amount of free glucose present was determined with the tris glucose oxidase reagent as described earlier (Dahlqvist and Thomson 1963 a) Lactose and galactose do not react with this reagent

Determination of lactose This was assayed by determining the increase in free glucose after the hydrolysis of the lactose by a fungal lactase preparation (Lactase F obtained from Koninklijke Gist en Spiritusfabriek N V Delft Holland) A buffered lactase solution was prepared by shaking 25 mg of the lactase preparation with 50 ml of 0 1 M

potassium[1] phosphate buffer pH 6 8 (6 95 g of KH_2PO_4 and 8 54 g of K_2HPO_4 dissolved in distilled water to 1 000 ml) for 5 min and then filtering When stored at + 4 C this solution is stable for at least 3 days To assay lactose in the protein free filtrate of the homogenates of the gastrointestinal tract 0 01 – 0 10 ml (containing 0 01 to 0 10 mg of lactose) was diluted with water to 0 1 ml then 0 4 ml of the buffered lactase preparation added and the tubes incubated at 37 C for 1 hour During this time the lactose was completely hydrolysed Then 3 0 ml of the tris-glucose oxidase reagent (Dahlqvist 1961) was added the tube again incubated at 37° C for 1 hour for development of the colour and the amount of glucose present after lactase digestion measured in the spectrophotometer by the method used for the assay of free glucose. To check that the lactose was hydrolysed completely a lactose solution of known concentration was always assayed together with the animal samples The amount of glucose liberated from lactose was calculated as the amount of free glucose found in the assay described above minus the amount of free glucose before lactase digestion The amount of lactose was then calculated by multiplication with a factor of 2

Determination of free galactose To determine the amount of free galactose the total amount of sugar present (lactose plus glucose plus galactose) was first determined with a reducing sugar method after the lactose had been split enzymatically Then the amount of free galactose originally present was calculated by subtracting the amounts of lactose plus free glucose found with the methods described above The lactase digestion and reducing sugar assay were performed as follows 0 05 – 1 0 ml of the protein free filtrate was diluted to 1 0 ml with distilled water 1 0 ml of the buffered lactase solution was added, and the tube then incubated at 37 C for 1 hour Then 2 0 ml of the 3 5-dinitrosalicylate reagent of Sumner (1924) prepared as described by Hostettler Borel and Deuel (1951) was added the tube heated in a boiling water bath for 10 min and then cooled with tap water After dilution with 20 ml of distilled water the colour was determined in a spectrophotometer at a wave length of 530 mμ in cuvettes with a 1 cm light pathway For zero-setting of the spectrophotometer a blank without sugar was used and the amount of monosaccharides was calculated from a standard series with 0 5 – 2 0 mg of glucose Glucose and galactose have the same extinction coefficient with this method

Specificity and accuracy of the analytical methods When known amounts of lactose glucose and galactose were mixed with segments of the small intestine from fasted rats and then homogenised and analysed by the above methods for the separate determination of each of these sugars 97 – 103 of the expected amounts of the sugars were found The recovery of polyethylene glycol has been discussed previously (Dahlqvist and Thomson 1963 a)

Calculation of the results of the absorption tests The calculations and the terms used for the results of the absorption experiments have all been defined previously (Dahlqvist and Thomson 1963 a and b) The statistical calculations used were performed according to Bailey (1959) The abbreviations used are S D = standard deviation and S E = standard error of the mean

Determination of the rate of hydrolysis of lactose in vitro by preparations from the different parts of the gastrointestinal tract of the rat Fasted rats were killed by a blow on the head and the gastrointestinal tract removed and divided as follows stomach upper middle and lower thirds of the small intestine caecum and the remainder of the large intestine Each segment was placed in a small beaker and weighed The caecal contents were first removed with a spatula and transferred to a separate beaker but the other segments were not opened The contents of each beaker were homogenized in an Ultra Turrax homogenizer with 4 ml/g of ice-cold 0 9 per cent NaCl The tube was chilled with crushed ice during the homogenization The homogenates were *not* centrifuged since in preliminary experi

[1] This fungal lactase is activated by potassium ions and inhibited by sodium ions

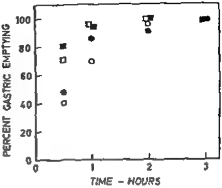

Fig 1 Gastric emptying calculated from the amount of polyethylene glycol recovered from the stomach at different times after the administration of the sugar polyethylene glycol solution. Each point represents the mean value obtained from two or more rats ● = 800 mg lactose fed ○ = 800 mg glucose + galactose fed, ■ = 400 mg lactose fed □ = 400 mg glucose + galactose fed

ments centrifugation was found to remove part of the lactase activity in accord with the results of Doell and Kretchmer (1962). The homogenates were assayed for lactase with the method of Dahlqvist (1960) using the tris buffered glucose oxidase reagent (Dahlqvist 1961). To reduce the turbidity of the solutions used for the spectrophotometric measurements the lactase reaction in the assay was interrupted by the addition of 0.1 ml of $ZnSO_4$ solution and 0.1 ml of $Ba(OH)_2$ solution adjusted to give an ion free supernatant (Somogyi 1945) instead of by boiling. Then the solution was diluted to 1.0 ml and after centrifugation 0.5 ml of the supernatant was taken for glucose assay as usual. One unit of lactase is the activity hydrolysing 1.0 mg of lactose monohydrate in 60 min under the specified conditions[1]

Rat pancreatic juice was obtained by cannulation of the lower end of the common bile pancreatic duct with polyethylene tubing. Bile was removed through another polyethylene catheter in the common bile duct near the liver to avoid contamination. The operation was performed under ether narcosis and the animal was then placed in a restraining cage and the pancreatic juice collected for 24 hours in tubes chilled with solid carbon dioxide.

Determination of the influence of pH on the lactase activity of homogenates of small intestinal mucosa and caecal contents The small intestinal mucosa of fasted rats was scraped off with a glass slide, weighed and homogenised with 4 ml/g of ice cold 0.9 per cent NaCl in the Ultra Turrax homogenizer. The tube was chilled with ice during homogenisation. The caecal contents in another tube were homogenised in the same way. Lactase assay was then performed on these homogenates with the usual procedure using the following buffers: pH 4–5.5 0.05 M sodium acetate, pH 6–7 0.05 M sodium maleate, pH 7.5 0.025 M sodium veronal. The buffer concentrations given are those in the reaction mixture. A separate set of tubes with the same reaction mixture was used for the assay of pH with a glass electrode both before and after incubation. The mean value of these measurements was used in plotting the pH/activity curves.

Determination of the rate of hydrolysis of lactose in vivo The amount of lactose hydrolysed at any given time in the absorption tests was calculated using the assumption that all of the lactose that had disappeared from the gastrointestinal tract after intubation had been absorbed and hydrolysed during its absorption. The amount was thus calculated as the

[1] In recent publications the unit for disaccharidase activity has been defined as the activity hydrolysing 1 µmole of substrate per min in accord with international agreements for enzyme units (Freeman 1961). In the present study the older lactase unit has been used in order to facilitate the comparison of rate of hydrolysis of lactose *in vitro* with its rate of hydrolysis and absorption *in vivo*

Table I Mean transit of the sugar polyethylene glycol solutions through the small intestine at various times after intragastric administration

Time after intubation (hrs)	No of animals	Sugar fed	Amount (mg)	Small intestine Upper third Mean	Upper third Range	Middle third Mean	Middle third Range	Lower third Mean	Lower third Range
0 5	3	Glucose + Galactose	800	64	45–75	28	20–33	0	—
	2	Lactose	800	79	75–83	47	36–58	5	0–11
	2	Glucose + Galactose	400	76	72–80	41	32–50	4	0–8
	2	Lactose	400	79	72–87	40	35–45	3	0–7
1 0	5	Glucose + Galactose	800	84	75–91	53	40–70	2	0–10
	2	Lactose	800	91	90–93	69	61–77	73	70–77
	2	Glucose + Galactose	400	97	96–98	91	—	3	2–5
	2	Lactose	400	97	90–95	53	50–57	5	—
2 0	3	Glucose + Galactose	800	97	95–98	84	76–97	3	2–3
	2	Lactose	800	95	94–96	69	59–79	54	40–68
	2	Glucose + Galactose	400	99	98–100	94	87–100	41	26–57
	2	Lactose	400	96	94–98	61	53–69	30	17–4[illegible]
4 0	2	Glucose + Galactose	800	98	—	98	96–100	60	51–70
	2	Lactose	800	100	—	100	—	78	76–81
	2	Glucose + Galactose	800	100	—	96	94–98	99	98–100
[illegible]	2	Lactose	800	100	—	98	—	94	91–97
[illegible]0	2	Glucose + Galactose	800	98	97–100	98	97–100	100	—
	2	Lactose	800	97	97–98	92	87–98	86	82–91

amount of sugar which had disappeared from the gastrointestinal tract plus the amount of free glucose and galactose recovered from all of the segments The rate of hydrolysis of lactose *in vivo* (mg/hr) was calculated from the values obtained during the first 3 hrs after intubation

Results

Gastric emptying In spite of the difference in molarity of the solutions very little difference was observed in the rate of gastric emptying after the administration of lactose or the corresponding amount of glucose and galactose (Fig 1) When 400 mg of sugar were given the stomach emptied more rapidly than when the larger dose was used Gastric emptying was over 95 % complete in two hrs

Transit through the small intestine No consistent effect on the rate of transit of the sugar polyethylene glycol solutions through the upper two-thirds of the

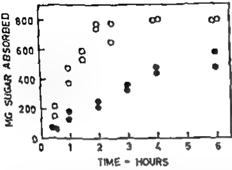

Fig 2

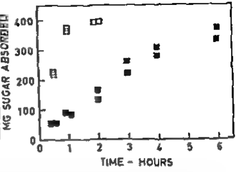

Fig 3

Fig 2 Total amount of sugar absorbed at different times after the intragastric administration of 800 mg of lactose (●) or 800 mg of an equimolar mixture of glucose and galactose (○)

Fig 3 Total amount of sugar absorbed at different times after the intragastric administration of 400 mg of lactose (■) or 400 mg of an equimolar mixture of glucose and galactose (□)

small intestine was observed after lactose administration (Table I) When 800 mg of lactose were given transit through the lower third of the small intestine was definitely more rapid than in the other cases No diarrhea occurred after lactose administration and the 800 mg dose used by us is well below the amount previous authors have found necessary to produce diarrhea (Fischer and Sutton 1957 Coryell and Christman 1943) Several rats were kept in individual metabolic cages for 12—18 hrs after the feeding of 800 mg of lactose and after this time the stools were solid and contained only 10—20 % of the polyethylene glycol given The remainder of the marker was recovered from the large intestine

Rate of absorption of the sugars Absorption proceeded practically linearly for 2 hrs when 800 mg of the glucose galactose mixture were given during which time over 90 % of the sugar was absorbed (Fig 2) During this period the monosaccharides were absorbed at the rate of 376 ± 26 mg/rat/hr (mean ± S E) When 400 mg of the monosaccharides were given 407 ± 21 mg/rat/hr of sugar were absorbed (Fig 3) Absorption was completed in 2—3 hrs when 800 mg of the monosaccharides were given and after 1—2 hrs when the lower dose was used Glucose was absorbed somewhat more rapidly than galactose when their rates of absorbed rates of absorption was calculated separately in agreement with the results of previous workers who have studied the absorption of a mixture of these two monosaccharides (Cori 1926 Coryell and Christman 1943)

When either 800 or 400 mg of lactose were administered absorption took place much more slowly than when the monosaccharides were given (Fig 2 and 3) In both cases the rate of absorption was virtually constant for 4 hrs while 60—75 % of the sugar was absorbed and then the rate decreased The 800 mg of lactose were completely absorbed in 8—12 hrs the 400 mg in about 6 hrs

Table II. Mean absorption indices for glucose + galactose and for lactose for the various segments at various time intervals after intubation

Time after intubation (hrs)	No. of animals	Sugar fed	Dose (mg)	Stomach Mean	Stomach Range	Small intestine Upper third Mean	Upper third Range	Middle third Mean	Middle third Range	Lower third Mean	Lower third Range	Large intestine Mean	Large intestine Range
0.5	3	Glucose+ Galactose	800	6	4–9	[illegible]	0–34	65	61–74	75	–	–	–
	2	Lactose	800	9	3–16	[illegible]	–	8	0–17	7	4–11	–	–
	2	Glucose+ Galactose	400	2	0–4	44	31–58	81	79–83	91	8[illegible]–97	–	–
	2	Lactose	400	0	–	6	0–13	11	11–12	11	–	–	–
1.0	5	Glucose+ Galactose	800	1	0–4	16	0–23	74	67–89	8[illegible]	79–88	–	–
	2	Lactose	800	9	–	–	–	2[illegible]	24–27	10	5–16	24	21–27
	2	Glucose+ Galactose	400	–	–	–	–	–	–	99	–	–	–
	2	Lactose	400	0	–	–	–	17	17–18	18	16–20	–	–
2.0	3	Glucose+ Galactose	800	–	–	–	–	88	82–94	98	96–100	–	–
	2	Lactose	800	–	–	–	–	30	21–39	25	15–34	22	0–44
	2	Glucose+ Galactose	400	–	–	–	–	96	–	100	–	100	–
	2	Lactose	400	–	–		–	42	29–55	39	37–41	38	–
3.0	2	Lactose	800	–	–			37	31–43	38	36–41	[illegible]1	–
	2	Lactose	400	–	–	–		75	–	66	64–68	70	54–8[illegible]
4.0	[illegible]	Lactose	800	–	–		–	–	–	60	53–67	57	4[illegible]–[illegible]0
	2	Lactose	400	–	–	–	–	–		71	6[illegible]–77	84	77–91
6.0	2	Lactose	800	–	–	–	–	–	–	71	–	66	60–73
	2	Lactose	400	–	–	–	–	–		96	9[illegible]–100	91	8[illegible]–97
8.0	2	Lactose	800	–	–	–	–	90	–	9[illegible]	–	74	71–[illegible]7
12.0	1	Lactose	800	–	–	–	–	–	–	–	–	100	–

Absorption indices were only calculated for those segments in which 10% (5 mg) or more of the administered polyethylene glycol was recovered.

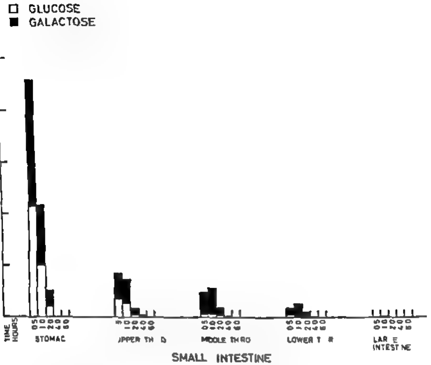

Fig 4 Mean amounts of glucose and galactose recovered from the different parts of the gastrointestinal tract at various t mes after the intragastric administration of 800 mg of an equimolar mixture of the s two sugars

During the linear absorption period the 800 mg dose was absorbed at the rate of 142 $\pm$ 15 mg/rat/hr (mean $\pm$ S E) and the 400 mg dose at the rate of 86 $\pm$ 5 mg/rat/hr The difference between these two means is significant at the 1 % level

Gastric and intestinal absorption indices Very little gastric absorption of the sugars occurred (Table II) When glucose and galactose were fed almost all of the sugar was absorbed in the upper two-thirds of the small intestine and after one hr over 95 % of the monosaccharides had been absorbed from the portions of the solution fed which had reached the lower third of the small intestine

Lactose absorption took place in all segments of the small intestine After the feeding of 800 mg of the disaccharide more than 60 % of the sugar remained in that portion of the solution given which reached the large intestine in the first two hrs When 400 mg of lactose were given small intestinal absorption proceeded more nearly to completion than after the larger dose

Over 90 % of the lactose was absorbed after 6 hrs when 400 mg were given and after 8—12 hrs when the 800 mg dose was used Since less than 20 % of the

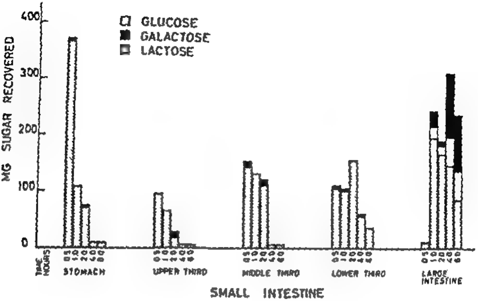

Fig 5 Mean amounts of glucose galactose and lactose recovered at various times after the intra gastric administration of 800 mg of lactose

amount of polyethylene glycol given was recovered from the stools in 12—18 hrs and since virtually no sugar was found in the stools after this time, almost all of the disaccharide was absorbed The amount of sugar utilized by the intestinal flora cannot be estimated in these experiments

Amount and type of sugar recovered from the various segments of the gastrointestinal tract When 600 mg of the glucose galactose mixture were fed no sugar was recovered from the large intestine and very little sugar even reached the lower third of the small gut (Fig 4) The amount of galactose recovered in any small intestinal segment always exceeded the amount of glucose

Large amounts of sugar reached the lower third of the small bowel and the colon when 800 mg of lactose were given (Fig 5) Over 25 % of the amount of sugar fed was found in the colon even after 6 hr In the samples recovered from the small intestine nearly all of the sugar was present as the disaccharide showing that the rate of absorption of glucose and galactose exceeds the rate of hydrolysis of lactose at this site Glucose and galactose accumulated to a considerable extent in the colon reflecting the much slower rate of absorption of these monosaccharides at this level of the intestinal tract When the 400 mg dose of lactose was fed a smaller fraction of the sugar was recovered from the colon

The localization of lactase in the rat intestinal tract The distribution of the total lactase activity of the gastrointestinal tract and caecal contents of the rat measured *in vitro* is seen in Table III Virtually no gastric lactase was found

Table III Lactase activity of the various segments of the gastro-intestinal tract and the caecal contents of the rat measured in vitro in homogenates

Segment	Units of lactase activity
Stomach	0 6
Upper third small intestine	35 6
Middle third, small intestine	49 6
Lower third small intestine	25 1
Caecal contents	86 7
Caecal wall	2? 7
Colon and rectum	6 4
Total activity	226 7

The small intestine of a rat is able to hydrolyse about 110 mg of lactose/hr under the conditions of the *in vitro* assay The higher lactase activity of the middle as compared to the proximal third of the small intestine is similar to the findings of Cajori (1935) in the dog The very high lactase activity of the ceacal contents is striking This is especially noteworthy in that the other disaccharidases are essentially absent from the caecal contents of the rat (Dahlqvist 1963) The caecal wall also shows some lactase activity but this may well be a result of contamination with caecal-content lactase Rat pancreatic juice containing 40 mg of protein/ml and with a high amylase activity did not contain lactase (less than 0 1 units/ml)

The locus and rate of lactose hydrolysis in vivo Parenterally injected lactose is largely excreted in the urine (Verzar and McDougall 1936) After the intra gastric administration of 800 mg of lactose to rats we collected the urine for up to 8 hrs The urine contained very little sugar (less than 20 mg) Thus in our experiments little or no lactose escaped hydrolysis in the gastrointestinal tract during its absorption That fraction of the dose of lactose which is absorbed in the small intestine can be hydrolysed by the amount of lactase we found in the small intestinal mucosa Since a considerable fraction of unhydrolysed lactose reaches the large intestine when 800 mg are given and since this fraction is split to monosaccharides and eventually absorbed the lactase of the caecal contents also seems to participate in the physiological digestion of this dose of lactose in the rat

The course of the hydrolysis of lactose after feeding 800 and 400 mg is seen in Fig 6 From the values for the first 3 hrs it was calculated that the rate of hydrolysis *in vivo* was 169 ± 13 mg/rat/hr (mean ± S E) when 800 mg of lactose were fed and 120 ± 10 mg/rat hr when 400 mg were given

The difference between these two values is significant at the 1 % level

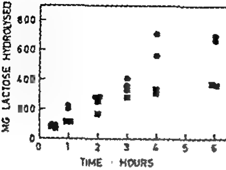

Fig 6 The course of the hydrolysis of lactose after the intragastric administration of 800 mg (●) or 400 mg (■) of this disaccharide

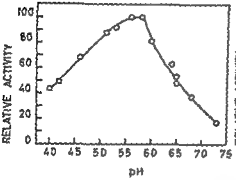

Fig 7

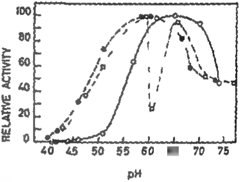

Fig 8

Fig 7 Influence of pH on the lactase activity of a homogenate of rat small intestinal mucosa

Fig 8 Influence of pH on the lactase activity of the caecal contents of the rat Three separate experiments each using different symbols are shown In each experiment the mixed caecal contents from two rats were used

Since the total lactase activity of the gastrointestinal tract of the rat was found to be 227 units representing the hydrolysis of 227 mg of lactose/hr (Table III) the observed rate of hydrolysis found *in vivo* can easily be accounted for by the amount of enzyme available

The origin of the caecal content lactase The remarkably high lactase activity of the caecal contents may be caused either by enzyme released from desquamated small intestinal mucosal cells or by bacterial enzymes To investigate these possibilities we studied the pH activity curves of the lactase found in small intestinal mucosal homogenates and homogenized caecal contents

In repeated experiments the pH-optimum for the small intestinal enzyme was found to be 5 5—5 8 The pH activity curve is seen in Fig 7

The pH-optimum of the lactase activity of the caecal contents varied from experiment to experiment (Fig 8) In one case the enzyme showed a pH-optimum considerably higher than the small intestinal enzyme (about pH 6 5) in a second experiment the optimum was closer to the small intestinal one and

in a third experiment two distinct optima were present These findings indicate that at least a considerable fraction of the caecal content lactase is enzymatically different from the small intestinal enzyme Since the caecal wall has a low lactase activity the second enzyme in the caecal contents is most probably of bacterial origin This concept is supported by the fact that practically no lactase is found in the caecal contents of germ free rats (Dahlqvist Bull and Gustafsson — to be published)

The pH optimum found for the rat small intestinal mucosal lactase agrees well with that found for intestinal lactase from other mammalian species (Heilskov 1956 Dahlqvist 1960) Heilskov (1956) has also shown that the pH optimum for the lactase in extracts from *E coli* is considerably higher than for the animal enzyme

Discussion

Lactose is known to exert a laxative effect both in man and animals (Fischer and Sutton 1949) With the doses of lactose we used however little effect on the gastrointestinal motility was observed The only clearcut effect the more rapid passage of lactose from the lower part of the small intestine into the colon when the 800 mg dose was fed can be explained on the basis of the osmotic effect of the large amount of sugar reaching the lower part of the small bowel in this case This finding supports the conclusions of earlier authors (Fischer 1955)

In absorption studies in man the absorption of lactose has been found to occur in the upper part of the small intestine (Dahlqvist and Borgstrom 1961) In the present investigation lactose was absorbed at all levels of the small bowel in the rat and particularly with the larger dose of lactose a considerable amount of the sugar was absorbed in the colon As lactase was present both in the whole of the small intestine and in the contents of the caecum it may well be that the major site of absorption found in a particular experiment is a reflection of the amount of sugar fed With the 800 mg dose in our experiments the capacity of the rat small intestine to hydrolyse lactose was considerably exceeded and at least 25 % of the sugar fed reached and was absorbed in the large intestine Also the ability of the small intestine of the adult rat to hydrolyse lactose as compared with its ability to hydrolyse other disaccharides may be relatively lower than that of the human intestine Human jejunal mucosa possesses a lactase activity comparable to that of invertase (Dahlqvist 1962) The small intestinal lactase activity of the adult rat found in this study is less than one third of its invertase activity (Dahlqvist and Thomson 1963 a) This relative lack of small intestinal lactase in the rat may partly explain the lower locus of lactose absorption found in this species

The importance of the lactase of the caecal contents seems to have received little attention in the past (see review by Fischer and Sutton 1949) The high lactase activity we found in the caecal contents of the rat seems to be caused by bacterial enzymes As all of our rats received the same diet which contained

5 1 % lactose the effect of the diet on the lactase activity of the caecal contents cannot be determined in these studies When large amounts of lactose are present in the diet the lactase of the caecal contents may well play a considerable role in the normal digestion and absorption of lactose

Lactose is known to have a specific enhancing effect on the absorption of calcium and other alkaline earth metals from the intestine (Irving 1957) After studying the absorption of radiostrontium in adult rats Cramer and Copp (1959) concluded that the ileum was quantitatively the most important site of absorption They also found that absorption continued for at least 4—6 hrs after the administration of the isotope Although the mechanism of the effect of lactose on the absorption of these metals has not been established the chelation of the metal ions by lactose has been proposed to be responsible for at least part of this effect (Charley and Saltman 1963) Our finding of large amounts of lactose in the lower third of the small intestine for up to 8 hrs after its administration shows that chelation could occur at this site throughout the time that these metals are being absorbed

The finding that unhydrolysed lactose reaches the colon fits well with the known influence of this disaccharide on the large intestinal bacterial flora (Fischer and Sutton 1949)

Grants that have been obtained from the Swedish Medical Research Council are gratefully acknowledged Mrs A Karstensson and Miss K Klang are thanked for skillful technical assistance

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Vascular Adjustments to Increased Transmural Pressure in Cat and Man with Special Reference to Shifts in Capillary Fluid Transfer

By

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Received 9 September 1963

Abstract

MELLANDER S B ÖBERG and H ODELRAM *Vascular adjustments to increased transmural pressure in cat and man with special reference to shifts in capillary fluid transfer* Acta physiol scand 1964 *61* 34–48 — When a vascular bed is exposed to an increased transmural pressure as occurs in the lower extremities in man on standing adjustments of the vascular tone take place which tend to protect the organism against excessive transcapillary loss of circulating fluid into the extravascular space Thus first by closure of a number of the precapillary sphincter vessels the size of the capillary surface area directly available for blood flow and hence for fluid exchange is reduced and the rate of filtration correspondingly decreased Second by an increase of the ratio of precapillary to postcapillary flow resistance which increases the pressure drop across the precapillary resistance vessels the rise in capillary pressure will be less than expected from the observed increase in arterial and venous pressures From a quantitative point of view the reduction of the capillary surface area seems to be by far the more important protective mechanism as it was shown that in the human foot the rate of filtration per unit transcapillary pressure difference was decreased to only 1/3 to 1/8 of normal when the regional transmural pressure was increased by shifting from the supine to the erect posture

The vascular response patterns described are evidently brought into play mainly by a reinforcement of the local inherent automaticity of the vascular smooth muscle induced by the raised transmural pressure but in all probability can be intensified to some extent by superimposed extrinsic vasomotor fiber influence This active vascular adaptation to hydrostatic load seems to be of greater importance than extravascular mechanisms for preventing edema formation in dependent regions in normal subjects during quiet standing

A pronounced increase of the hydrostatic blood pressure in the lower extremities will occur in man on shifting from the supine to the erect posture Provided that a continuous column of blood from the heart level to the feet exists

not only on the arterial but also on the venous side of the circulation (see Pollack and Wood 1949 Hojensgård and Stürup 1952) and no counteracting mechanisms are brought into play the arterial venous and capillary pressures in the foot of the adult human should increase by about 75 to 85 mm Hg on standing Such a hydrostatic load will considerably affect the hemodynamics of the dependent vascular circuits In addition to the pronounced accumulation of blood that would occur in the highly distensible capacitance vessels , regional hydrostatic capillary pressure would greatly exceed plasma colloid osmotic pressure causing a rapid outward filtration of intravascular fluid with a consequent tendency for edema formation Using the data for the capillary filtration coefficient (CFC) in the human forearm given by Landis and Gibbon (1933), (0 005—0 006 ml fluid filtered/min/100 g tissue/mm Hg capillary pressure rise) it can be calculated that in the erect position the fluid loss into the tissue spaces in the human feet would amount to 0 5 to 1 liter/hour It is obvious that edema formation of this magnitude does not take place in healthy subjects in the erect posture (Turner Newton and Haynes 1930 Waterfield 1931) It must therefore be assumed that some potent mechanisms that protect against excessive fluid loss are brought into play on standing but the exact nature of such mechanisms does not seem to be fully understood at present Factors of importance in this connection would seem to be an increase of tissue pressure an augmentation of lymph drainage from the tissues and the action of the muscle pump lowering the venous pressure and hence also the capillary pressure No doubt these mechanisms may be operating to prevent edema in dependent regions during active muscle movements such as walking as will be discussed below During quiet standing however such factors do not seem to play an important role since then tissue pressure appears to be only moderately changed (e g Wells Youmans and Miller 1938 Day Hinshaw and Walder 1960) and regional venous pressure approaches that expected from the magnitude of the hydrostatic load (Pollack and Wood 1949 Hojensgård and Sturup 1952) and finally lymph drainage is then very small (see White Field and Drinker 1933 Yoffey and Courtice 1956)

It seems however quite possible that adjustments of regional vascular tone reflexly and/or locally induced can contribute to the limitation of transcapillary fluid loss in a region exposed to raised intravascular pressure for the following reasons The rate of the net filtration into the tissues is ultimately dependent upon the extent to which mean capillary pressure is raised and upon the size of the capillary surface area that is available for filtration One of the main determinants of capillary pressure is the ratio of precapillary to postcapillary resistance It is known that activation of the vascular smooth muscle generally leads to an increase of this ratio which per se tends to reduce mean capillary pressure (Mellander 1960) Furthermore changes of smooth muscle tone in the precapillary sphincters will determine the size of the capillary surface area that in a given situation is directly available for blood flow and therefore to

transcapillary fluid exchange (Folkow and Mellander 1960 Cobbold *et al* 1963) It seems likely that on shifting from the supine to the erect position there will be an increased vascular tone in dependent regions partly due to reflex vasoconstrictor fiber excitation and partly due to reinforcement of the inherent smooth muscle automaticity consequent upon the raised transmural pressure (see Folkow 1962) If now in the erect position such changes in vascular tone occur and these lead to an increased pre /postcapillary resistance ratio and/or to a closure of a fraction of the precapillary sphincters then these adjustments would tend to reduce outward filtration by limiting the capillary pressure increase and by reducing the capillary surface area available for exchange

In the present investigation quantitative methods have been used to follow the vascular reactions and the rate of net capillary fluid transfer occurring in a region exposed to raised intravascular pressure Special attention has been paid to the problem of whether and to what extent the size of the capillary surface area and hence the tone of the precapillary sphincters might be changed in such circumstances A quantitative measure of the size of the capillary surface area was assessed by the determination of the capillary filtration coefficient (CFC), as described by Cobbold *et al* (1963)

In one series of experiments such studies were made in the human foot with the subject in supine and erect body position Another series of experiments was performed on cats in which it was possible to follow in much more detail the vascular adjustments induced by raised transmural pressure In this preparation it was also possible to study separately the role of local mechanisms in terms of *intrinsic myogenic factors in these vascular adjustments*

Methods

General considerations

A technique which permits simultaneous recording of the reactions in different series coupled sections as well as net transcapillary fluid shifts in a vascular bed has been described previously (Mellander 1960) In the present study this technique was modified so as to permit recordings in the human foot and in a calf muscle-skin preparation *in the cat* The reactions of the resistance vessels could be assessed by recording arterial inflow pressure venous outflow pressure and regional blood flow By plethysmographic *volume recording of the organ it was possible to follow continuously both* variations in regional blood content (capacitance function) and changes in net fluid transfer across the capillary membrane Changes in the size of the capillary surface area and hence changes in the tone of the precapillary sphincters were followed by determinations of CFC (Cobbold *et al* 1963) CFC was determined by raising the venous outflow pressure a known amount and recording the rate of outward filtration caused by the consequent increase in capillary hydrostatic pressure (see Pappenheimer and Soto-Rivera 1948 Mellander 1960) To estimate to what extent mean hydrostatic capillary pressure was affected by a given venous pressure increase the pre /postcapillary resistance ratio must be known (Cobbold *et al* 1963) Therefore an evaluation of this ratio was done in these studies whenever CFC was to be determined

Experimental procedures

a *Cat experiments*

The vascular reactions to increased intravascular pressure were studied in a calf muscle skin preparation in 5 cats anesthetized with chloralose (40 mg/kg) and urethane (50 mg/kg). The calf preparation was done according to the method described by Kjellmer (1961). After removal of the paw the calf was placed in a water filled temperature-controlled plethysmograph using a skin flap for water proof closure. After heparinization the cognate artery and vein were severed and then reconnected to the femoral artery and vein respectively by means of fairly long siliconized polyethylene tubing. In this way the calf was perfused with normal oxygenated blood from the main part of the animal. The calf was then separated completely from the animal with careful hemostasis. The animal was placed on a table which could be elevated some 65—70 cm above the level of the isolated denervated calf in the plethysmograph. By adjusting the height of the table this preparation made it possible to follow the vascular reactions in the calf during normal and greatly increased transmural pressures but with the perfusion pressure head remaining constant. The arterial inflow pressure and venous outflow pressure in the calf were continuously recorded at the level of the plethysmograph. Calf blood flow was continuously measured by a drop recorder unit inserted on the venous side. With the plethysmographic technique changes in tissue volume were recorded continuously. CFC was determined at intervals.

In this way the reactions of the abovementioned vascular sections were followed when the vascular bed was exposed to normal and increased transmural pressures. After this chloral (0.5 g in 20 ml Dextran Tyrode solution) was slowly infused intra arterially into the calf to abolish the inherent activity of the vascular smooth muscles remaining after vasomotor denervation (Folkow and Löfving 1956). The behaviour of the vascular bed during normal and high transmural pressure was then studied.

b *Human experiments*

Experiments were performed on 8 healthy young men. The subject was placed on a tilt table with his right foot enclosed in a water filled temperature-controlled (35 C) plethysmograph fixed to the table. A thin latex stocking covering the foot was fixed to the entrance of the plethysmograph to ensure water tight closure. In the erect posture the weight was borne entirely by the left foot while the right foot was held at rest comfortably without loading. Room temperature was kept at 20 C. The water level in the plethysmograph was kept constant in both the supine and the erect position. Changes of foot volume were recorded continuously by means of a piston volume recorder connected to the plethysmograph. For determination of CFC venous outflow pressure in the foot was raised 20 to 30 mm Hg above the resting value. This was accomplished by instantaneous inflation of a blood pressure cuff placed around the calf and connected to a pressure container. Venous outflow pressure was measured continuously by means of an indwelling catheter in a vein on the dorsum of the right foot, the tip of which was placed inside the plethysmograph and directed centrally. Venous pressure was recorded with a strain gauge transducer connected to a direct writing oscillograph (Mingograph, Elema Co.), the transducer being placed at the level of the tip of catheter in the vein. Clotting was prevented by flushing the catheter at intervals with 2 heparin in saline. Arterial inflow pressure was measured in the popliteal fossa of the left leg according to Riva Rocci's method. Functional mean arterial pressure was taken as the diastolic pressure plus 1/3 of the pulse pressure. Blood flow in the foot was determined by venous occlusion plethysmography.

In each experiment CFC and foot blood flow were first measured repeatedly in the supine position. After this the subject was tilted to a head up position 80 from the

transcapillary fluid exchange (Folkow and Mellander 1960, Cobbold *et al* 1963) It seems likely that on shifting from the supine to the erect position there will be an increased vascular tone in dependent regions partly due to reflex vasoconstrictor fiber excitation and partly due to reinforcement of the inherent smooth muscle automaticity consequent upon the raised transmural pressure (see Folkow 1962) If now in the erect position such changes in vascular tone occur and these lead to an increased pre /postcapillary resistance ratio and/or to a closure of a fraction of the precapillary sphincters then these adjustments would tend to reduce outward filtration by limiting the capillary pressure increase and by reducing the capillary surface area available for exchange

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In this way the reactions of the abovementioned vascular sections were followed when the vascular bed was exposed to normal and increased transmural pressures After this chloral (0 5 g in 20 ml Dextran Tyrode solution) was slowly infused intra arterially into the calf to abolish the inherent activity of the vascular smooth muscles remaining after vasomotor denervation (Folkow and Lofving 1956) The behaviour of the vascular bed during normal and high transmural pressure was then studied

b *Human experiments*

Experiments were performed on 8 healthy young men The subject was placed on a tilt table with his right foot enclosed in a water filled temperature controlled (35 C) plethysmograph fixed to the table A thin latex stocking covering the foot was fixed to the entrance of the plethysmograph to ensure water tight closure In the erect posture the weight was borne entirely by the left foot while the right foot was held at rest comfortably without loading Room temperature was kept at 20 C The water level in the plethysmograph was kept constant in both the supine and the erect position. Changes of foot volume were recorded continuously by means of a piston volume recorder connected to the plethysmograph For determination of CFC venous outflow pressure in the foot was raised 20 to 30 mm Hg above the resting value This was accomplished by instantaneous inflation of a blood pressure cuff placed around the calf and connected to a pressure container Venous outflow pressure was measured continuously by means of an indwelling catheter in a vein on the dorsum of the right foot the tip of which was placed inside the plethysmograph and directed centrally Venous pressure was recorded with a strain gauge transducer connected to a direct writing oscillograph (Mingograph Elema Co) the transducer being placed at the level of the tip of catheter in the vein Clotting was prevented by flushing the catheter at interval with 2 % heparin in saline Arterial inflow pressure was measured in the popliteal fossa of the left leg according to Riva Rocci s method Functional mean arterial pressure was taken as the diastolic pressure plus 1/3 of the pulse pressure Blood flow in the foot was determined by venous occlusion plethysmography

In each experiment CFC and foot blood flow were first measured repeatedly in the supine position After this the subject was tilted to a head up posi ion 80 from the

horizontal plane CFC and blood flow were then recorded repeatedly in the erect position The rate of fluid accumulation in the foot occurring spontaneously during standing was then recorded for periods of 15—30 min Finally the subject was put back in the supine position followed again by repeated determinations of CFC and blood flow The foot volume was determined at the end of the experiment Since volume changes can occur *only in tissues outside the skeleton the weight of the bone was excluded in the calcula*tions of CFC The skeleton approached roughly 40 % of the total weight of the region studied to judge from the wet weight of foot skeletons from adult humans

In an attempt to study more separately the importance of local intrinsic mechanisms in the vascular response pattern produced by raised transmural pressure an additional series of experiments was performed in which reflex vasoconstrictor nerve influence could be essentially avoided The subject was lying flat on this back first with his right leg stretched so that the foot in the plethysmograph was kept at the same horizontal plane as the trunk Then his leg was bent at the knee joint over the edge of the table and therefore the vascular bed of the foot exposed to an increased hydrostatic load corresponding to a column of blood extending from the knee to the foot CFC was determined in the two different positions of the foot Since some pooling of blood will occur in the leg exposed to increased hydrostatic pressure which reflexly might engage the vasoconstrictor fibers the amount of blood pooled was kept essentially constant during the whole experiment by shifting the position of the other leg opposite to that of the leg studied

Results

a *Cat experiments*

Fig 1 illustrates a representative experiment in which arterial inflow pressure, venous outflow pressure blood flow and volume changes in the calf preparation were recorded In A and B the basal vascular tone was maintained i e one remaining after complete vasomotor denervation and presumably due mainly to the spontaneous inherent myogenic activity of the vascular smooth muscle (Folkow 1962) In C and D this basal tone was largely abolished by chloral administration The perfusion pressure gradient across the vascular bed was essentially the same in all four sections of this figure while the transmural pressure was normal in A and C and was greatly elevated in B and D by increasing both arterial inflow pressure and venous outflow pressure by about 50 mm Hg The calculated values for regional peripheral resistance (Green 1948) are indicated in the figure (PRU) as well as the values found for the filtration coefficient

Comparison of section A and D in Fig 1 demonstrates that an increase in the transmural pressure (B) increases PRU from 14 6 to 17 2 apparently due to reinforcement of the myogenic activity of the vascular smooth muscles in the resistance vessels Folkow 1962) In addition the raised transmural pressure produced a slight distension of the capacitance vessels (mainly postcapillary vessels) *which was shown by a rapid increase in tissue volume (not shown in the* figure) If the raised transmural pressure had produced a reinforcement of the myogenic activity of the capacitance vessels also this would have been noted as a secondary decrease of volume after the initial passive increase This did however not occur which indicates that a significant augmentation of the myo-

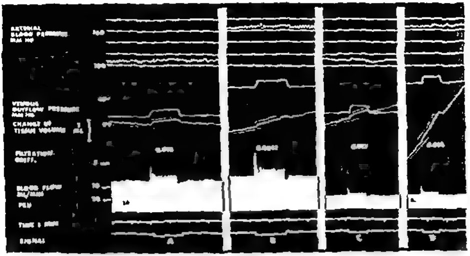

Fig 1 Vascular reactions in a skin muscle preparation (weight 130 g) in cat during "normal" (A and C) and elevated (B and D) transmural pressure the perfusion pressure kept constant In A and B the "basal" tone was maintained in C and D it was abolished by chloral At signals, venous outflow pressure is elevated for determination of CFC.

genic activity in the postcapillary vessels did not take place Therefore the increase in the flow resistance produced by the raised transmural pressure seems to be almost completely confined to precapillary resistance vessels It can then be calculated that the pre /postcapillary resistance ratio has shifted from 4/1 in A (taken from the data of Pappenheimer and Soto-Rivera 1948 and Cobbold *et al* 1963) to 5/1 in B (or expressed in PRU from 11 7/2 9 to 14 3/2 9) CFC was determined in A and B (signals) using the above mentioned ratios in the calculation It was found that CFC was reduced to less than half the original value (by about 60 %) when the transmural pressure was elevated indicating a corresponding decrease of the capillary surface area directly available for exchange

The present study also made it possible to deduce approximately the extent to which mean hydrostatic capillary pressure and effective filtration pressure (i e mean hydrostatic capillary pressure minus tissue pressure) were increased in this vascular region when the transmural pressure was raised from A to B With a pre /postcapillary resistance ratio of 5/1 and an increase of arterial and venous pressures of 55 and 50 mm Hg respectively in B the elevation of mean hydrostatic capillary pressure can be calculated to amount to 48 mm Hg (see Renkin and Pappenheimer 1957) If the ratio had remained at 4/1 this pressure increase would have been 51 mm Hg Thus the increase of pre- postcapillary resistance ratio during this hydrostatic load implies that the capillary pressure increase is reduced by some 5 to 10 % As can be seen in Fig 1 B the rise of transmural pressure produces a continuous gain of tissue volume owing to an increase of

effective outward filtration pressure and the consequent transcapillary fluid filtration from the circulation into the extravascular space When the rate of this outward filtration and the value for CFC are known, the rise in effective filtration pressure can be calculated It amounted in this experiment to 47 mm Hg which is in close agreement with the value for the mean hydrostatic capillary pressure increase of 48 mm Hg calculated above From this it seems reasonable to conclude that tissue pressure and plasma and tissue colloid osmotic pressures were hardly at all affected during the period of raised transmural pressure

When chloral was given so as to considerably decrease or eliminate the inherent activity of the vascular smooth muscle there was a marked dilatation of the resistance vessels (PRU decreased from 14 6 to 6 3), but a very small dilatation of the capacitance vessels (not shown in the figure) This again suggests that basal tone is confined almost exclusively to precapillary resistance vessels as discussed above Following the slight capacitance response there was a continuous gain of tissue volume indicating an increased mean capillary hydrostatic pressure and consequent outward filtration of fluid (C in Fig 1) Since arterial and venous pressures were kept constant this capillary pressure rise must be due to a decreased pre /postcapillary resistance ratio Under the present conditions with abolished vascular reactivity this ratio could be determined according to Pappenheimer and Soto Rivera (1948) It was then found that the outward filtration caused by increasing the venous outflow pressure 10 mm Hg was counterbalanced by lowering the arterial inflow pressure about 20 mm Hg implying a /postcapillary resistance ratio of 2/1 — CFC determined in C amounted to 1 017, indicating in comparison with A an increased functionally available capillary surface area due to relaxation of precapillary sphincters In D the transmural pressure was raised to the same level as in B In contrast the results obtained before chloral administration regional peripheral resistance decreased somewhat when transmural pressure was raised obviously due to a passive elastic distension of the vessels Since now virtually no active adjustments of vascular tone within the resistance vessels seem to occur upon elevation of the transmural pressure the pre /postcapillary resistance ratio in D was considered to be the same as in C CFC was then found to be 0 016 or roughly the same as in C indicating that now also the tone of precapillary sphincters was largely unaffected by the elevated transmural pressure The fact that CFC was reduced during increased transmural pressure when basal tone was maintained (B) but unchanged when it was abolished (D) clearly demonstrates that the reduction of CFC obtained in B was due to an increased locally induced activity in the precapillary sphincter vessels

It can be seen in the figure that the rate of outward filtration (gain of tissue volume) is considerably greater in D than in B due partly to the greater CFC and partly to a more pronounced increase in capillary pressure consequent to the reduced pre /postcapillary resistance ratio The capillary pressure was thus

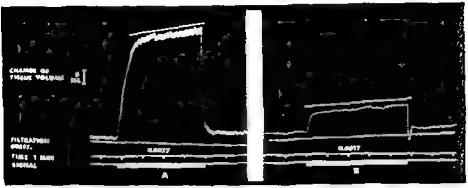

Fig 2 Recording of volume changes in the human foot At signals venous outflow pressure is raised for determination of CFC during the supine posture (A) and the erect posture (B) Note that CFC decreases markedly during standing

calculated to increase by 52 mm in D as compared with 48 mm Hg in B the rise of arterial and venous pressure being largely the same in the two cases

The rise of effective filtration pressure in D as calculated from the rate of outward filtration and CFC amounted however to only 49 mm Hg This difference of 3 mm Hg between the rise of capillary hydrostatic pressure and effective filtration pressure might be ascribed to a corresponding increase in tissue pressure due to a gradual accumulation of extravascular fluid A tissue pressure increase of this magnitude does not seem unlikely as it could be estimated that up to this point of the experiment roughly 15 ml fluid corresponding to about 10 % of the initial tissue weight had accumulated in the tissue spaces

The results reported above can be considered representative for the whole series of cat experiments since the data obtained in the other animals were quantitatively approximately the same as those described above

b *Experiments on the human foot*

Fig 2 shows a representative experiment in which the CFC was determined in supine (A) and erect position (B) In A (signal) cuff pressure was raised so as to increase venous pressure in the foot 24 mm Hg This resulted in an initial rapid increase in foot volume due to accumulation of blood in the capacitance vessels amounting to almost 40 ml After this there followed a continuous slow volume increase due to outward filtration (cf Mellander 1960) The pre-/postcapillary resistance ratio under these resting conditions was assumed to be 4/1 The CFC was then found to be 0 0077 ml/min/100 g/mm Hg This value is in good agreement with the data on filtration rate in the human forearm given by Landis and Gibbon (1933) After release of the cuff pressure tissue volume was restored to the initial level Note that before and after the CFC determination the volume curve shows a slight decline (dashed line at bottom)

which is interpreted as the effect of a spontaneously occurring slow continuous inward filtration of extravascular fluid into the circulation due to the external pressure of the water in the plethysmograph (cf Mellander 1960) Blood flow in the supine position determined by venous occlusion plethysmography amounted to 4 0 ml/100 g/min From this value and the prevailing mean arterial inflow pressure (90 mm Hg) and venous outflow pressure (36 mm Hg) regional peripheral resistance (PRU) was calculated to be 13 5 The fact that resting venous pressure was relatively high is in all probability dependent upon the external pressure on the foot exerted by the water in the plethysmograph as will be discussed below When the subject was tilted to the standing position there was an abrupt and fairly large increase in foot volume due to regional accumulation of blood secondary to the regional hydrostatic load Section B of Fig 2 shows the volume recording a few minutes after the tilting Note that there is now a spontaneous slow upward deflection of the volume curve (bottom dashed line) indicating a continuous filtration of fluid from the circulation to the extravascular space This recording provided direct information about the net fluid loss from the circulation during standing The rate of this extravascular fluid accumulation amounted to 0 067 ml/100 g tissue/min or 40 ml/kg tissue/h At the signal CFC was determined in the same way as in A the increase in venous pressure here amounting to 22 mm Hg The initial volume increase due to filling of the capacitance vessels was considerably ller than in supine position about 8 ml, probably partly due to the prevailing distension of the capacitance vessels caused by accumulation of blood on tilting the subject and partly to a compensatory reflex increase in venous smooth muscle tone (Page *et al* 1956) In the erect po ition CFC was found to be only 0 0017 or 4 5 times smaller than in the supine position The pre /post capillary resistance ratio used for this calculation was 4/1 Blood flow determined in the erect position amounted to 3 9 ml/100 g/min Mean arterial inflow pressure was now found to be 170 mm Hg and venous outflow pressure 84 mm Hg and therefore PRU 21 5 or about 60 % higher than in the supine position Such a change implies an increase in the pre /postcapillary resistance ratio to greater than 4/1 which in turn means that the CFC would be even somewhat smaller in standing position than calculated here as will be discussed below A characteristic change in the volume recording was noted on shift from supine to erect position In the supine position there were volume oscillations of fairly great amplitude synchronous with the arterial pulse As is seen in B these oscillations were almost completely abolished in the upright position This may reflect an altered distensibility of the Windkessel vessels possibly due to a reflex change in their smooth muscle tone and/or to the distension caused by the raised transmural pressure The somewhat larger irregular variations in the volume curve in both A and B are probably due to spontaneous changes in the tone of the capacitance vessels and the consequent minor changes in regional blood volume

Fig 3 Change of CFC in the foot of 8 adult human subjects on shifting from the supine to the erect posture Each value represents a mean of 4 to 6 determinations

Fig 3 shows the changes of CFC in the foot obtained in the 8 subjects when shifted from supine to erect position Each value plotted is a mean of 4 to 6 determinations in the same subject It can be seen that as a rule CFC decreased to 1/3 to 1/6 of control when the subject was tilted into the erect position The values for the CFC in a given position varied to some extent in the individual subject but on no single occasion during standing did the CFC amount to more than 2/5 of control

In all experiments venous pressure in the superficial foot vein rose in the erect position to values corresponding to a pressure exerted by a column of blood extending from the right atrium to the site of recording as long as no limb movements were done by the subject

In the series of experiments in which flexion of the knee joint with the subject remaining in supine position was performed the vascular bed of the foot was exposed to a hydrostatic load of about 35 mm Hg No change in vasomotor fiber discharge seemed to take place during this procedure to judge from the fact that no increase of heart rate was observed CFC was found to decrease to about 1/3 of control during this hydrostatic load This strongly suggests that the reduction in CFC obtained during raised transmural pressure to a great extent is dependent upon a reinforcement of the local inherent myogenic activity and that this change of CFC is not necessarily due to an increased sympathetic vasomotor fiber influence

Discussion

The rate of fluid filtration from the circulation to the extravascular space in a region exposed to a high intravascular hydrostatic pressure is above all dependent upon the extent to which effective filtration pressure is raised and upon the size of the capillary surface area that is available for filtration The present experiments have demonstrated that both these variables can be affected by changes of regional vascular smooth muscle tone elicited during hydrostatic load In this way an effective protection can be established against excessive loss of circulatory fluid into tissues at rest The most potent protective mechanism seems to be accomplished by closure of a fraction of the regional preca

which is interpreted as the effect of a spontaneously occurring slow continuous inward filtration of extravascular fluid into the circulation due to the external pressure of the water in the plethysmograph (cf Mellander 1960) Blood flow in the supine position determined by venous occlusion plethysmography amounted to 4 0 ml/100 g/min From this value and the prevailing mean arterial inflow pressure (90 mm Hg) and venous outflow pressure (36 mm Hg) regional peripheral resistance (PRU) was calculated to be 13 5 The fact that resting venous pressure was relatively high is in all probability dependent upon the external pressure on the foot exerted by the water in the plethysmograph, as will be discussed below When the subject was tilted to the standing position there was an abrupt and fairly large increase in foot volume due to *regional accumulation of blood secondary to the regional hydrostatic load* Section B of Fig 2 shows the volume recording a few minutes after the tilting Note that there is now a spontaneous slow upward deflection of the volume curve (bottom dashed line) indicating a continuous filtration of fluid from the circulation to the extravascular space This recording provided direct information about the net fluid loss from the circulation during standing The rate of this extravascular fluid accumulation amounted to 0 067 ml/100 g tissue/min or 40 ml/kg tissue/h At the signal CFC was determined in the same way as in A the increase in venous pressure here amounting to 22 mm Hg The initial volume increase due to filling of the capacitance vessels was considerably aller than in supine position about 8 ml probably partly due to the prevailing distension of the capacitance vessels caused by accumulation of blood on tilting the subject and partly to a compensatory reflex increase in venous smooth muscle tone (Page *et al* 1956) In the erect position, CFC was found to be only 0 0017 or 4 5 times smaller than in the supine position The pre /post capillary resistance ratio used for this calculation was 4/1 Blood flow determined in the erect position amounted to 3 9 ml/100 g/min Mean arterial inflow pressure was now found to be 170 mm Hg and venous outflow pressure 84 mm Hg and therefore PRU 21 5 or about 60 % higher than in the supine position Such a change implies an increase in the pre /postcapillary resistance ratio to greater than 4/1 which in turn means that the CFC would be even somewhat smaller in standing position than calculated here as will be discussed below A characteristic change in the volume recording was noted on shift from supine to erect position In the supine position there were volume oscillations of fairly great amplitude synchronous with the arterial pulse As is seen in B these oscillations were almost completely abolished in the upright position This may reflect an altered distensibility of the Windkessel vessels possibly due to a reflex change in their smooth muscle tone and/or to the distension caused by the raised transmural pressure The somewhat larger irregular variations in the volume curve in both A and B are probably due to spontaneous changes in the tone of the capacitance vessels and the consequent minor changes in regional blood volume

The quantitative significance of the abovementioned smooth muscle adjustments for the net capillary fluid transfer in a vascular circuit exposed to high transmural pressure is clearly demonstrated in the experiment on a human presented in Fig 2 If none of the abovementioned vascular adjustments had taken place when the subject was tilted into the erect position CFC and pre/postcapillary resistance ratio would remain about the same as in the recumbent position (0 0077 and 4/1 respectively) The increase in mean capillary hydrostatic pressure in the foot on standing would then amount to roughly 55 mm Hg calculated from the recorded increase in arterial and venous pressures (see Renkin and Pappenheimer 1957) Using these values for capillary pressure increase and CFC the net outward filtration in the foot would then have amounted to 255 ml fluid/kg tissue/h This hypothetical value greatly exceeds the amount of fluid that in reality was found to be accumulated in the foot on standing (40 ml/kg/h) Owing to vasomotor reactions produced during the increased hydrostatic intravascular pressure on standing CFC was reduced to 0 0017(using pre /postcapillary resistance ratio 4/1) It can then be calculated that this mechanism by itself will limit the net transcapillary fluid filtration to 55 ml/kg/h which only slightly exceeds the value of fluid accumulation actually found (40 ml) This small discrepancy might partly be due to the fact that the pre /postcapillary resistance ratio actually does increase on standing leading to a less pronounced rise of capillary pressure than assumed and also to a CFC slightly below 0 0017 Reliable quantitative data on shifts in this ratio could not be obtained in the present experiments on man The fact that resistance to blood flow increased about 60 % in the foot supports however the assumption that the pre /postcapillary resistance ratio is increased on standing since it is known that a neurogenically induced increase of flow resistance probably here reinforced by a myogenic effect always is associated with an increased ratio (Mellander 1960)

The small discrepancy between the above calculated (55 ml/kg/h) and observed (40 ml/kg/h) fluid loss into the tissues could also be attributed to other protective mechanisms against fluid loss such as the action of the muscle pump tending to lower the venous and thereby the capillary pressure an increased tissue pressure tending to reduce effective filtration pressure or an increased lymph drainage from the region It is evident from the above calculation that during brief quiet standing all these factors are of little quantitative importance as compared to the great limitation of transcapillary fluid loss caused by precapillary sphincter closure reducing the CFC

The fact that the resting venous pressure in the foot in the supine position was relatively high (25 to 35 mm Hg) deserves some comments In these experiments an external pressure on the foot was created by the water in the plethysmograph and also perhaps to some extent by the latex stocking To keep the veins patent the intravascular pressure must exceed this external pressure (compare the so-called water fall phenomenon) (Permutt Bromberger Barnea and Bane 1962)

described in kidneys (Swann, Montgomery and Lowry 1951) and in plethysmographic preparations (Kjellmer 1963) The rise in venous pressure to about 85 mm Hg on standing will due to this high resting venous pressure, imply an effective pressure increase of only 50 to 55 mm Hg Under normal conditions therefore the effective increase in venous and capillary pressures on standing can be expected to be greater than in the present experiments and the accumulation of fluid consequently somewhat greater than estimated above

It should be emphasized that the observed net accumulation of fluid in the resting foot on standing (40 ml/kg/h) by no means can be considered insignificant It is probably responsible for the slight edema seen in the feet on prolonged quiet standing and in dependent immobilized limbs The situation is in all likelihood quite different when active muscle movement takes place in the dependent limbs Under such circumstances, the extent of fluid accumulation might be much less than at complete rest The pumping action of the skeletal muscles then tends to reduce the pressure increase in veins with competent valves (Pollack and Wood 1949) thereby limiting the increase of mean capillary pressure also It is also possible that tissue pressure rises to higher levels under these circumstances, reducing effective filtration pressure Finally lymph drainage is increased to some extent by active muscle movements (Jacobsson and Kjellmer 1963) which will reduce net fluid accumulation in the tissues

Of the two abovementioned vascular adjustments which in this study have been shown to be engaged in counter balancing outward filtration during hydrostatic load i e a reduction of the capillary surface area by sphincter ure and a reduction of the capillary pressure increase by raised pre /post illary resistance ratio the first mechanism seems to be not only more effective but also more suitable from the hemodynamic point of view If the rise of capillary pressure were limited to a greater extent by an increase in the pre/postcapillary resistance ratio there would be such a pronounced increase in the total resistance to blood flow that the nutrition of the tissue might be jeopardized It has been shown previously that in skeletal muscle an increase of this ratio leading to a fall of capillary hydrostatic pressure of 10 mm Hg is associated with a total flow resistance increase of 500 to 800 % (Mellander 1960) On the other hand a reduction in the size of the capillary surface area by closure of a fraction of the precapillary sphincters causes only insignificant changes of total flow resistance in the region due to the low pressure gradient across this particular vascular section Closure of a number of these sphincters per se, will therefore hardly interfere with the volume flow of blood but it implies that the available blood is diverted through a smaller number of capillaries It is true that flow velocity in patent capillaries then must be increased shortening the time available for exchange and that the diffusion distance to the tissue cells may be prolonged temporarily Since however the rate of diffusion for most relevant molecules is extremely high (Pappenheimer 1953) a moderate decrease of the capillary surface area will not necessarily cause any

significant impairment of tissue nutrition Furthermore there are reasons to believe that the individual sphincters constrict temporarily and out of phase with each other so that blood is alternated through different capillary channels The functional net effect still will be a reduction of the capillary surface area (Cobbold *et al* 1963), but blood flow will become more evenly distributed within the capillary network

It is of interest in this connection to note the results of Prerovsky *et al* (1962) who found that the clearance of subcutaneously injected I^{131} decreased markedly in skin tissue in the human foot on shifting from the supine to the standing position This was interpreted as a decrease of capillary blood flow In view of the present study it seems likely that an additional explanation for the decreased clearance would be that on standing the size of the capillary surface area available for exchange is diminished

To judge from the present experiments it seems that the described vascular smooth muscle adjustments following raised transmural pressure to a great extent depend upon a reinforcement of the inherent myogenic activity of the vasculature This seems to be a well suited regulatory mechanism since the vascular adjustments protecting against excessive loss of circulating fluid will be most powerful in the very regions where the increase of hydrostatic pressure and the consequent tendency for outward filtration are most pronounced That such a local regulatory mechanism is operating may be further supported by the findings of Prerovsky *et al* (1962) that I^{131} clearance was more reduced in distal than proximal parts of the human leg skin on standing There is no doubt that extrinsic vasoconstrictor fiber influence elicited on shift from supine to erect position may intensify the abovementioned vascular adjustments This may however more generally engage the vascular regions in the organism regardless of site and hydrostatic load since no evidence is as yet available that selective activation of the vasoconstrictor fibers in dependent parts alone will occur in such situations The functional significance of increased vasomotor fiber discharge on standing may therefore not primarily be to protect against transcapillary fluid loss in dependent regions but rather to produce more generalized hemodynamic adjustments to maintain cardiovascular homeostasis For example nervous influence in contrast to myogenic reactions produces a constriction of the capacitance vessels which will limit accumulation of blood in dependent regions and thereby help to maintain venous return in the erect position

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Further Studies on the Action of Thyroid Hormones on the Metabolism of Collagen

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Abstract

KIVIRIKKO K I M KOIVUSALO and O LAITINEN *Further studies on the action of thyroid hormones on the metabolism of collagen* Acta physiol scand 1964 *61* 49—54 — In experiments on rats the effects of triiodo-L-thyronine and of hypothyroidism induced either by propylthiouracil or ^{131}I on the urinary excretion of hydroxyproline were studied Further the content of 0 45 M NaCl soluble collagen hydroxyproline in the skin was determined in the experiment on hypothyroidism In experiments with chick embryo the effect of the propylthiouracil treatment on free hydroxyproline and 1 M NaCl-soluble collagen hydroxyproline was studied The amounts of both free and total hydroxyproline excreted in the urine of rats increased after the administration of triiodo-L-thyronine and decreased greatly after the administration of propylthiouracil or ^{131}I In the skin of rats given ^{131}I or propylthiouracil the content of 0 45 M NaCl soluble collagen hydroxyproline diminished but the decrease after propylthiouracil was not statistically significant In chick embryos the relative amounts of free hydroxyproline and of 1 M NaCl-soluble collagen hydroxyproline expressed as a percentage of the total hydroxyproline and the total amounts of hydroxyproline per embryo were lower in the propylthiouracil treated embryos than in the controls

Our earlier study (Kivirikko et al 1963) indicated that the excretion of hydroxyproline in the urine of rats increased after the administration of L-thyroxine The experiments were continued by studying the effect of triiodo-L-thyronine and of hypothyroidism induced by propylthiouracil or ^{131}I on the urinary excretion of hydroxyproline in rats In the hypothyroid groups the content of neutral salt soluble collagen hydroxyproline in the skin was also determined Furthermore the effect of propylthiouracil on the relative amounts of free hydroxyproline and neutral salt soluble collagen hydroxyproline expressed as a percentage of the total hydroxyproline was studied in chick embryos

Table I The effects of triiodo L thyronine on the urinary excretions of free and total hydroxyproline and on the weight of the rats The hydroxyproline values are expressed as μ per 24 hours (Mean ± SD) the body weight in grams (Mean ± SD)

Determination	Group	Initial value	After 5 days	After 9 days
Weight	Controls	222 ± 16	230 ± 18	245 ± 17
	Triiodothyronine	223 ± 15	212 ± 20	208 ± 24
Free hydroxyproline	Controls	81 7 ± 22	63 8 ± 31	71 6 ± 20
	Triiodothyronine	64 0 ± 14	74 9 ± 18	108 3 ± 22
Total hydroxyproline	Controls	588 ± 155	484 ± 171	520 ± 111
	Triiodothyronine	555 ± 118	634 ± 138	900 ± 219

7 rats in each group

After 9 days the value for P was $P < 0\ 01$ both in free hydroxyproline and in total hydroxyproline when the values of the treated group were compared with those of the controls by the *t* test

Material and methods

Experiments with rats

The test animals were male albino Wistar rats which were 2 1/2—3 months old at the beginning of the study Two series of experiments were made

In the first series the effect of daily intraperitoneal administration of 20 μg 3 5 triiodo-L-thyronine (Nutritional Biochemicals Corporation) on the urinary excretion of free and total hydroxyproline was studied

In the second series one group of rats received 0 075 per cent 4 (6) propyl 2 thiouracil (Eli Lilly and Company) in their drinking water and another group 1 mC per rat of ^{131}I at the start of the experiment (day 0) and a further 0 5 mC per rat 10 days later A third group served as controls Urine was collected before the start of the experiment and thereafter at 20-day intervals until the 100th day and further at the 140th day Two of the propylthiouracil treated rats died after the 100th day of the experiment After 140 days the animals were killed and a piece of their dorsal skin was removed and analysed for 0 45 M sodium chloride soluble collagen hydroxyproline

Collection of the urine and preparation of the 0 45 M sodium chloride soluble collagen fractions of the skin were carried out as in our earlier study (Kivirikko *et al* 1963)

Experiments with chick embryos

4 (6) Propyl 2 thiouracil was injected daily in 0 2 ml of 0 9 per cent sodium chloride *into the air space of embryonated eggs on the 9th—15th days of incubation* The daily doses were adjusted to the daily weights of the embryos known from an earlier study (Kivirikko 1963) The daily dose was 50 μg/g and the total dose given during 7 days 2 mg

After 16 days incubation the embryos were removed from the eggs weighed rapidly and homogenized in cold (± 0 C) 1 M NaCl 2 ml/g wet weight of the embryo The homogenates were fractioned as described earlier (Kivirikko 1963) with slight modifications and analysed for free hydroxyproline 1 M sodium chloride soluble collagen hydroxyproline and total hydroxyproline

Hydroxyproline was determined in all experiments by the method of Prockop and Udenfriend (1960) but the volumes of all the reagents used were reduced to one-half

Table II The effect of propylthiouracil (PTU) and of ^{131}I *on the urinary excretions of free and total hydroxyproline and on the weight of the rats The hydroxyproline values are expressed as µg/24 hours the body weight in grams Mean values are given and in the initial and final values the standard deviations also*

Determination	Group	Initial value	After 20 days	After 40 days	After 60 days	After 80 days	After 100 days	After 140 days
Weight	Controls	213±8	274	280	293	299	300	342±34
	PTU	210±13	237	209	196	183	—	214±21
	^{131}I	208±14	245	257	2[illegible]0	231	270	299±53
Free hydroxyproline	Controls	46.7±17.6	50.7	34.8	31.8	29.7	35.0	40.9±8.7
	PTU	39.9±14.6	28.2	22.7	16.3	14.6	—	11.4±2.1
	^{131}I	53.1±20.2	47.8	21.5	20.8	20.0	27.2	17.3±6.8
Total hydroxyproline	Controls	456±169	443	361	350	274	378	367±64
	PTU	513±106	247	146	134	106	—	107±37
	^{131}I	503±89	312	215	213	210	174	172±47

7 rats in the control and ^{131}I groups 6 in the PTU group after 100 days 4 in the PTU group

After 140 days the value for P was < 0.001 both in free hydroxyproline and in total hydroxyproline when the values of the PTU treated or ^{131}I treated groups were compared with those of the controls by the *t* test

Results

Experiments with rats

The effect of triiodothyronine (Table I) — There was some decrease in the weights of the rats receiving 20 µg of triiodothyronine daily Increased amounts of both free and total hydroxyproline were excreted even after 5 injections and after 9 injections the increase was still greater

The effects of propylthiouracil and ^{131}I — During the course of the experiment there was only a slight retardation in the weight gain of the ^{131}I treated rats whilst the weight gain of the propylthiouracil treated rats was completely inhibited (Table II) The excretions of both free and total hydroxyproline decreased greatly in both groups during the course of the experiment Table II)

The content of hydroxyproline in the *0 45 M sodium chloride soluble collagen* fraction of the skin after the 140-day experiment was 595 ± 80 µg/g wet weight of the skin in the controls and 430 ± 64 ($p < 0.01$) µg g in the ^{131}I treated rats In the 4 propylthiouracil treated rats that were alive after 140 days this value was 502 ± 54 µg/g which did not differ significantly from that for the controls

Experiments with chick embryos

Two similar series of experiment were made The administration of propylthiouracil was followed by some retardation in the weight gain of the treated

Table III The effects of propylthiouracil on the hydroxyproline values wet weights and relative dry weights of 16 day-old chick embryos Propylthiouracil was given during the 9th—15th days of incubation The values are expressed as mean ± SD

Group	Weight of embryos (g)	Relative dry weight (mg/g wet weight)	Total hydroxy proline (mg per embryo)	Total hydroxy proline (µg/ 100 mg dry weight of embryo)	Free hydroxy proline (of total hydroxy proline)	1 M NaCl-sol coll hydroxy proline (of total hydroxy proline)
I Controls (6)	14 28 ± 1 31	165 ± 14	20 2 ± 3 2	858 ± 108	4 46 ± 1 05	6 62 ± 1 08
Propylthiouracil (6)	*11 95 ± 0 65	*148 ± 4	*16 9 ± 1 3	963 ± 130	*3 32 ± 0 48	5 54 ± 0 90
II Controls (7)	14 79 ± 1 15	162 ± 6	22 7 ± 1 1	953 ± 75	3 01 ± 0 42	6 37 ± 0 56
Propylthiouracil (7)	*12.87 ± 1 71	150 ± 11	20 9 ± 4 1	10 74 ± 70	*1 83 ± 0 38	*5 60 ± 0 72

Number of embryos within brackets.

The significance of the differences as compared with the controls is denoted by =P<0 05 †=P<0 01 =P<0 001

embryos (Table III) Furthermore the propylthiouracil treated embryos contained less dry substance per g wet weight than the controls The absolute amount of hydroxyproline per embryo was lower in the propylthiouracil treated embryos than in the controls but the concentration of hydroxyproline per unit dry weight was higher The relative amounts of free hydroxyproline and of 1 M sodium chloride-soluble collagen hydroxyproline were lower in the propylthiouracil treated embryos than in the controls

Discussion

The results of the present study indicate that the amounts of both free and total hydroxyproline excreted in the urine of rats are increased after the administration of triiodo-L-thyronine and decreased in hypothyroidism induced either by propylthiouracil or by ^{131}I The results are in agreement with those of our earlier study which indicated increased excretion of hydroxyproline in the urine of rats after the administration of L-thyroxine (Kivirikko *et al* 1963) In addition it has quite recently been reported that the excretion of hydroxy

proline in the urine is increased in patients with hyperthyroidism (Dull and Henneman 1963 Kivirikko *et al* 1964) and decreased in patients with hypothyroidism (Keiser and Sjoerdsma 1962) and children with cretinism (Jasin *et al* 1962) The finding that the concentration of hydroxyproline in the serum is also increased in patients with hyperthyroidism (Kivirikko *et al* 1964) indicates that the increased excretion in the urine is due not to renal changes but to changes in the metabolism of collagen

According to the present view collagen fibres are built up from tropocollagen molecules that are synthesized intracellularly and thereafter transported into the extracellular space where they combine to form fibres (for review see *e g* Kivirikko 1963) With neutral salt solutions it is possible to extract tropocollagen molecules that are not yet aggregated into fibres or that are only more or less loosely aggregated depending on the ionic strength of the salt solution used (see *e g* Jackson and Bentley 1960) Further it seems probable that in young animals a considerable part of the urinary hydroxyproline is derived from these soluble collagen fractions (*e g* Lindstedt and Prockop 1961, Jasin *et al* 1962 Jasin and Ziff 1962 Procop 1962) Therefore in our studies attempts have been made to determine changes in the content of the neutral salt soluble collagen fraction of the skin of rats These determinations have revealed a slight decrease in both hypothyroidism (this study) and hyperthyroidism (Kivirikko et al 1963)

Since the changes in the content of the neutral salt soluble collagen fraction were relatively slight it is not possible to draw definite conclusions as to their significance The decreased content of neutral salt soluble collagen hydroxyproline in the skin of young rats in hypothyroidism taken in conjunction with the decreased excretion of hydroxyproline in the urine suggests that the rates of formation and turnover of collagen are decreased in hypothyroidism The decreased content of soluble collagen in the skin of young rats in hyperthyroidism despite the increased excretion of hydroxyproline in the urine might be due to increased catabolism of the soluble collagen in this condition It is not possible to establish on the basis of these studies whether the catabolism of the insoluble collagen is also increased or whether the rate of formation of soluble collagen is altered in hyperthyroidism

The results obtained with chick embryos are in agreement with the suggestion that the rates of formation and turnover of collagen are decreased in hypothyroidism for the relative amounts of free hydroxyproline and 1 M sodium chloride soluble collagen hydroxyproline expressed as a percentage of the total hydroxyproline and the absolute amounts of total hydroxyproline per embryo were lower in the propylthiouracil treated embryos than in the controls The increased concentration of total hydroxyproline in the propylthiouracil treated embryos may be explained by assuming that there was an even greater decrease in the rate of formation of some other substances which would result in an increased concentration of collagen Since the effects of propylthiouracil were similar to

those of ^{131}I in the experiments with rats it seems probable that the effects observed in chick embryos were due to hypothyroidism However, a direct toxic effect of propylthiouracil on the metabolism of collagen in chick embryos can not be excluded

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Inhibition of Gastric Acid Response to Sham Feeding in Pavlov Pouch Dogs by Acidification of Antrum

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Abstract

ANDERSSON, S. and L. OLBE. *Inhibition of gastric acid response to sham feeding in Pavlov pouch dogs by acidification of antrum.* Acta physiol. scand. 1964. *61.* 55—64. — The nervous phase of gastric acid secretion was studied on sham fed Pavlov pouch dogs with an isolated, innervated antrum. The sham feeding response was not inhibited by antral acidification to pH > 2.5. A slight but highly significant inhibition occurred at antral pH 2 and a substantial inhibition at antral pH 1—1.5. The results suggest that on vagal stimulation gastrin is released from the antrum at antral pH ≧ 2 in amounts sufficient to augment significantly the nervous secretory response. Inhibition of the sham feeding response by antral acidification was eliminated by a very low submaximal dose of intravenously administered gastrin in accordance with the hypothesis that depression of vagal release of gastrin is the main inhibitory mechanism elicited by antral acidification during the nervous phase of gastric acid secretion.

Acidification of the antrum has been shown to inhibit substantially the gastric secretory response to insulin hypoglycemia in Pavlov pouch dogs (Shimizu, Morrison and Harrison 1958, Andersson 1960 a, Johnson Jr. *et al.* 1960, Thomson, Lerner and Tramontana 1962). In these studies the pH of the antral contents was usually reduced to 1.0—1.5 by instillation of 0.1 N HCl into the antrum. However, no attempts were made to determine the critical pH level at which the antral inhibitory mechanism is activated.

Several alternatives are available to explain the mechanism by which acidification of the antrum inhibits the nervous secretory response of the stomach: (1) Inhibition of vagal release of gastrin (Maung Pe Thein and Schofield 1959, Nyhus *et al.* 1960, Johnson Jr. *et al.* 1960). (2) Liberation of an inhibitory humoral agent from the antral mucosa (Shimizu, Morrison and Harrison 1958, State and Morgenstern 1958, Thompson *et al.* 1962). (3) An inhibitory reflex.

The purpose of the present study was to investigate the inhibitory influence of varying antral acidity on the nervous phase of gastric acid secretion and the mode of action of this inhibitory mechanism.

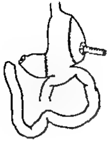


Fig 1 Operative procedure Pavlov pouch and innervated antral pouch

Methods

Eight healthy mongrel dogs (A B G—L) were provided with esophageal fistulas and innervated gastric (Pavlov) pouches Antrum-duodenum exclusion established by a double mucosal wall between the corpus and the antrum and pylorotomy with resection of the pyloric ring were later performed in all dogs The distal part of the innervated antrum was brought out to the abdominal wall (Fig 1) Gastrointestinal continuity was restored by gastrojejunostomy In the first 2 dogs operated upon (K L) the mucosal wall was placed on the presumed antrum corpus boundary according to anatomical criteria but in the remaining 6 dogs (A B G—J) on the antrum corpus boundary after localization of the distal border of the acid secreting mucosa according to a method described by Olbe (1963) The secretory responses to sham feeding were determined after each operation Operative and experimental procedures have been previously described in detail (Olbe 1959 1963)

Before each experiment the dogs fasted for 18—20 hours In each experiment the fasting Pavlov pouch secretion was first determined for at least one hour After sham feeding the secretion was collected for 3 1/2 hours The secretion was collected in 15 min periods and the amount of free and total acid was determined by titration against 0 01 N NaOH with Topfer s reagent and phenolphthalein as indicators In the tables and figures the secretory output is expressed in milliequivalents of total acid Instillation of solutions of varying pH into the innervated antral pouch generally started 15 min before sham feeding and proceeded for 3 3/4 hours In 2 dogs (A B) acid was introduced into the antrum also one hour after sham feeding The solutions used for antral irrigation included 0 1 N HCl 0 1 N HCl diluted with physiologic saline McIlwain citric acid buffer (pH 3) and Sorensen phosphate buffer (pH 6 7) The instillation was performed at a rate of 50—100 ml per hour The solution to be instilled was heated to 38 C and introduced into the deepest part of the antral pouch through a tube 3—4 mm in diameter In dogs A B G K and L the solution flowed from the antral pouch without obstruction In dogs H—J the technique was modified the solution flowed from the antral pouch through a tube the outlet of which was placed about 10 cm above the top of the antral pouch The solution from the antral pouch was collected every 15 min and its pH was determined electrometrically with a glass electrode

In two dogs (I J) gastrin was continuously infused i v throughout the sham feeding experiments while the intra antral pH was kept at 1 by instillation of acid against a pressure of 10 cm H_2O The i v infusion of gastrin was started 1 3/4 hours before acidification of the antrum Gastrin was administered in a dose of 6 7 (dog I) and 1 5 $\mu g/kg/$min (dog J) which was the lowest dose which by itself produced acid secretion The gastrin was prepared according to the method of Gregory and Tracy (1961) but the purification was stopped at stage one The gastrin preparations had a secretory activity of 106—120 histamine units (Uvnas and Emås 1961) per mg The histamine content of the gastrin preparations was less than 0 [illegible] μg free histamine base per mg gastrin

Table I Influence of intra antral pH on the 3.5 hours secretory response to sham feeding in Pavlov pouch dogs following antrum duodenum exclusion

Dog	Mean secretory response to sham feeding (Meq ± SE)[1]								
	Before exclusion	After exclusion with intra antral pH 6—7	After exclusion with concomitant antral acidification to intra antral pH 1.0—1.2	1.4—1.6	2.0—2.2	2.5—2.6	2.9—3.3	3.6—4.4	6.0—6.8
A	9.05 ± 0.21 (4)	9.67 ± 0.57 (7)	—	—	8.14 ± 0.58 (3)	—	12.03 ± 1.69 (4)	—	12.14 ± 0.37 (4)
B	1.05 ± 0.20 (4)	6.60 ± 0.31 (6)	1.85 ± 0.61 (2)	—	4.31 ± 0.32 (2)	6.56 ± 1.05 (2)	7.78 ± 0.09 (2)	—	6.48 ± 0.68 (4)
G	1.30 ± 0.17 (5)	7.58 ± 0.45 (6)	1.81 ± 0.25 (2)	—	5.99 ± 0.92 (2)	—	7.37 ± 0.77 (2)	—	7.29 ± 0.?7 (2)
H	0.48 ± 0.10 (5)	4.31 ± 0.77 (4)	0.55 ± 0.10 (3)	—	2.76 ± 0.49 (3)	—	3.92 ± 0.27 (2)	4.60 ± 1.15 (3)	—
I	0.49 ± 0.07 (5)	1.84 ± 0.28 (4)	0.54 ± 0.04 (6)	0.63 ± 0.04 (3)	1.21 ± 0.20 (2)	1.61 ± 0.45 (2)	2.21 ± 0.69 (4)	2.11 ± 0.16 (4)	—
	0.50 ± 0.07 (5)	3.96 ± 0.23 (4)	0.85 ± 0.18 (7)	0.88 ± 0.31 (3)	2.5? ± 0.56 (4)	—	3.9? ± 0.28 (5)	3.50 ± 0.23 (3)	—

[1] Figures within parenthesis refer to number of experiments
Intra antral pH 3—6

Results

A Post Exclusion Antral pH — The pH of the antral secretions during acid secretion by the pouch of the corpus was 6—7 in dogs A B H — J 3—6 in dog G 1.3—1.8 in dog L and 1.0—1.2 in dog K.

B Sham Feeding Responses At Different pH Levels In the Innervated Antrum — (Table I Fig 2) The secretory response to sham feeding in Pavlov pouch dogs is substantially increased following neutralization of the antral-duodenal milieu by antrum-duodenum exclusion (Olbe 1963) These hypersecretory sham feeding responses (dogs A B G—J) were not diminished by reduction of the antral pH to 2.5 At an antral pH of 2 the hypersecretion was partially inhibited (the difference between the sham feeding responses at neutral antral

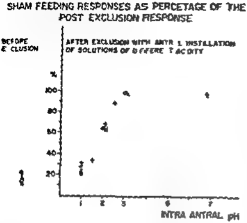
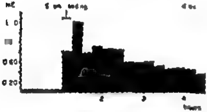


Fig 2 Mean acid secretory responses to sham feeding in Pavlov pouch dogs before and after antrum-duodenum exclusion with post-exclusion variation of intra antral pH as percentage of the post exclusion response Each symbol represents one dog



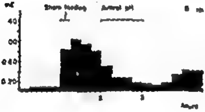


Fig 3 Mean acid secretory response to sham feeding in a Pavlov pouch dog following antrum-duodenum exclusion with and without antral instillation of acid during second hour after sham feeding (dog A)

milieu and those at antral pH of 2 was highly significant $p < 0.001$ according to analysis of variance) The reduction averaged 28 per cent At an antral pH of 1—1 5 the *hypersecretion was almost eliminated However the post exclusion* sham feeding responses at antral pH of 1 were still higher than those before exclusion (Table I) (the difference was significant $p < 0.01$)

In dogs H and J the inhibition of the sham feeding response at antral pH of 1 was only moderate when the acid flowed unobstructedly from the antrum mean sham feeding response was 1 61 meq (4 expts) and 2 73 meq (5 expts) respectively (cf Table I) However when the acid from the antrum flowed against a small pressure of 10 cm H_2O the inhibition of the sham feeding response in dogs H and J was of the same magnitude as that in the other dogs (Table I) The acid inhibition of the sham feeding response at antral pH of 1 in dog I was the same with or without the use of *outflow pressure of 10 cm H_2O in the antrum*

Table II Influence of intra antral pH on the secretory response to sham feeding in Pavlo pouch dogs following antrum duodenum exclusion with acid secreting mucosa left in the excluded antral pouch

	Mean secretory response to sham feeding (Meq ± SE)		
	1st hour	2nd hour	3rd hour
Dog K			
Before exclusion (4 exp)	1.84 ± 0.18	0.67 ± 0.07	0.71 ± 0.21
After exclusion (4 exp) intra antral pH 1.0–1.2	1.66 ± 0.29	1.15 ± 0.24	0.68 ± 0.16
Buffer solution of pH 6.7 in excluded antrum during 1st hour (4 exp)	4.09 ± 0.31	1.76 ± 0.30	0.85 ± 0.17
0.1 N HCl in excluded antrum during 1st hour (4 exp)	1.61 ± 0.16	1.06 ± 0.18	0.66 ± 0.18
Dog L			
Before exclusion (4 exp)	0.09 ± 0.03	0.11 ± 0.04	0.13 ± 0.01
After exclusion (4 exp) intra antral pH 1.3–1.8	0.30 ± 0.06	0.30 ± 0.05	0.29 ± 0.10
Buffer solution of pH 6.7 in excluded antrum during 1st hour (4 exp)	0.75 ± 0.03	0.56 ± 0.05	0.23 ± 0.08
0.1 N HCl in excluded antrum during 1st hour (4 exp)	0.21 ± 0.04	0.38 ± 0.14	0.19 ± 0.06

In dogs A and B the antral pH was reduced to 1 one hour after the sham feeding (9 expts) The hypersecretion was eliminated after about 15—30 min and reappeared gradually following cessation of the antral acidification (Fig 3)

Dogs K and L had a considerable amount of acid secreting mucosa in the excluded antrum resulting in an antral pH of 1 0—1 2 and 1 3—1 8 respectively during acid secretion in the corpus In these dogs the post exclusion increase of the sham feeding response was absent (dog K) or moderate (dog L) The post exclusion sham feeding responses were unchanged by instillation of acid of pH 1 into the antral pouches However the responses were substantially augmented by installation of buffer solution of pH 6 7 into the antra (Table II Fig 4)

C Sham Feeding Responses at Antral pH 1 During Concurrent Administration of Exogenous Gastrin in Minimal Secretory Dosage — In 2 dogs continuous i v infusion of gastrin alone resulted in a secretory response of 0 01—0 08 meq per 15 min with free acid appearing in 4 out of 6 expts The sham feeding response at antral pH 1 was considerably augmented by concurrent administration of these small amounts of gastrin and equalled (dog J) or exceeded (dog I) the sham feeding response at neutral antral milieu (Fig 5)

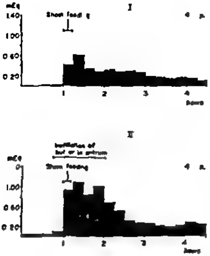


Fig 4 Influence of buffer instillation into innervated antral pouch on mean acid secretory response to sham feeding in a Pavlov pouch dog with acid secreting mucosa in the isolated antrum following antrum-duodenal exclusion (dog K)
I Intra antral pH 1.0—1.2 II Intra antral pH 5.2—6.5 during buffer instillation

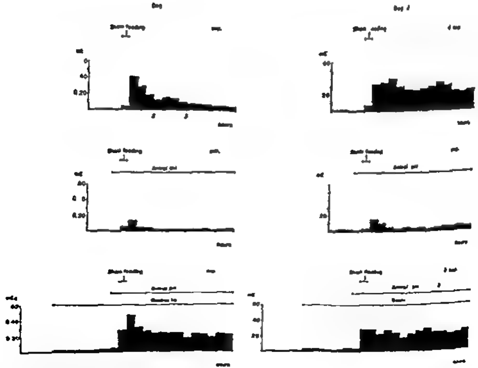


Fig 5 Effect of acidification of innervated antral pouch with and without concomitant administration of very low submaximal dose of exogenous gastrin on mean sham feeding response in Pavlov pouch dogs after antrum-duodenum exclusion

Discussion

The gastric acid secretory response to sham feeding in Pavlov pouch dogs is substantially augmented by antrum-duodenum exclusion (Olbe 1963). The hypersecretion was almost abolished by antral acidification to pH 1.0—1.5 in the present study, consistent with the marked inhibition of the secretory response to insulin hypoglycemia by antral perfusion with 0.1 N HCl which was observed by Shimizu, Morrison and Harrison (1958), Andersson (1960 a), Johnson Jr *et al.* (1960) and Thompson, Lerner and Tramontana (1962). Duodenal acidification as well as antral acidification has produced the marked inhibition of the secretory response to insulin hypoglycemia (Andersson 1960 a). The hypersecretory sham feeding response following antrum-duodenum exclusion in Pavlov pouch dogs is most probably due to elimination of acid inhibition from the antrum and duodenum. The mechanism of the hypersecretion probably involves augmented vagal release of gastrin from the neutralized antrum (Maung Pe Thein and Schofield 1959) and a subsequent synergism between the action of gastrin and vagal discharge on the HCl glands (Olbe 1963) in the absence of any acid inhibitory influence from the duodenum. The fact that Heidenhain pouch secretory response to i.v. administered gastrin is inhibited by duodenal acidification (Andersson 1960 b) emphasizes the significance of duodenal neutralization in the post exclusion hypersecretion of the present study but incomplete knowledge of antral and duodenal acid inhibitory mechanisms prevents satisfactory evaluation of the relative importance of antral vs duodenal neutralization.

Perfusion of the antrum with 0.1 N HCl inhibits Heidenhain pouch secretory response to sham feeding (Maung Pe Thein and Schofield 1959) but does not reduce its secretory response to large doses of i.v. administered gastrin (Gillespie and Grossman 1962). These findings suggest that antral acidification interferes with the vagal release of gastrin from the antrum rather than with the effect of gastrin on the HCl glands by a humoral inhibitor. The present observation that the inhibitory effect of low antral pH on sham feeding response in Pavlov pouch dogs is eliminated by small amounts of exogenous gastrin (Fig. 5) is further evidence against action of a chalone from the acidified antrum at parietal cell level. Another theoretically possible mechanism of acid inhibition from the antrum during the nervous phase of gastric acid secretion is reflex inhibition via afferent vagal fibres. Afferent impulses have been demonstrated in vagal fibres from the antrum on application of acid to the antral mucosa (Iggo 1957). However, when acid of pH 1 was introduced into the antrum during the course of a sham feeding response in the present study the inhibition required 15—30 min to develop and gradually disappeared after cessation of the antral acidification (Fig. 3). Such slow development and disappearance of the inhibition may be taken as evidence against a pure reflex mechanism. Furthermore, inhibition of the sham feeding response by antral acidification was eliminated by i.v.

administration of very low submaximal amounts of gastrin (Fig 5), which does not support reflex inhibition of vagally induced secretion by antral acidification The results speak in favour for depression of vagal release of gastrin as the main inhibitory mechanism elicited by antral acidification during the nervous phase of gastric acid secretion

During the course of the investigation we recognized the importance of filling the antrum with acid against a small outflow pressure in order to obtain maximal inhibition in each dog In 2 of the dogs (dogs H and J) simple instillation of acid of pH 1 into the deepest part of the antral pouch did not produce any pronounced inhibition of the sham feeding responses However, when the acid was instilled against a small outflow pressure (about 10 cm H_2O) in the antral pouch, the inhibition was of the same magnitude as in the other dogs (Table I) Apparently, in some dogs a slight pressure in the antrum is necessary to elicit acid inhibition An intermittent increase of pressure in the antrum operates during physiological conditions since intraluminal pressure waves of an amplitude of 5—10 cm H_2O occur in the antrum of fasting dogs and these waves are not influenced by sham feeding (Olbe and Jacobson 1963) One possible effect of increased pressure is to bring the acid into closer contact with pH sensitive receptors which may lie in the crypts of the antral mucosa

In 2 dogs (K and L) antrum duodenum exclusion was performed before development of the method for strict localization of the antrum corpus boundary during operation (Olbe 1963) In these dogs the antral pouch was found to contain acid secreting mucosa giving the antral secretion a pH of 1 0—1 8 following sham feeding The sham feeding response in these animals was unchanged (dog K) or moderately increased (dog L) by exclusion and the post exclusion sham feeding response was not further depressed by antral instillation of acid of pH 1 (Table II) The results suggest that continuous artificial acidification of the antrum starting immediately before vagal stimulation elicits no stronger inhibition than physiologic antral acidification by vagally induced gastric juice The inhibition evoked by artificial antral acidification in the present study therefore probably represents a physiologic mechanism In these dogs with acid secreting mucosa in the isolated antrum the post exclusion sham feeding response was markedly augmented by antral instillation of buffer solution of pH 6 7 (Fig 4) which corroborates the view that the increased nervous secretory response following antrum duodenum exclusion is a consequence of elimination of acid inhibition from the antrum and the duodenum

The hypersecretory sham feeding response following antrum duodenum exclusion was not reduced by antral acidification with a pH > 2 5 in the present study At antral pH 2 a considerable part of the hypersecretory response still persisted (Fig 2) although a highly significant inhibition occurred At antral pH 1 0—1 5 the hypersecretion was almost eliminated (Fig 2) The very small part of the hypersecretion that remained at antral pH 1 0—1 5 could possibly be due to the post-exclusion elimination of duodenal acid inhibition

The results suggest that during the nervous phase of gastric acid secretion a significant vagal release of gastrin from the antrum does occur as long as the antral pH $\geqq$ 2 However Nyhus *et al* (1960) found in Heidenhain pouch dogs that the secretory response to vagal release of gastrin was abolished by antral acidification to pH 2 It is possible that the amount of gastrin released from the antrum at pH 2 by vagal stimulation is insufficient to produce secretion from a Heidenhain pouch (Nyhus *et al* 1960) but is still sufficient to enhance the nervous secretory response via synergism with the vagal effect on the HCl glands in a Pavlov pouch (Olbe 1963) It has recently been demonstrated that the vagally innervated HCl glands are far more sensitive to gastrin than the vagally denervated glands (Andersson and Olbe 1964)

Very few data are available concerning the pH at which gastrin release from the antrum by mechanical and chemical means is inhibited Gastrin release by mechanical stimulation of the antrum is inhibited by antral acidification to pH 2 (Gregory and Tracy 1960) and is probably abolished by an antral pH between 1 3 and 1 7 (Woodward *et al* 1957) The critical pH for pronounced inhibition of chemical release of gastrin from the antrum is probably below 2 (Elwin 1960) The results of the present study suggest that vagal release of gastrin from the antrum is pH dependent in the same way as release of gastrin by other means indicating that the same mechanism may by responsible for acid inhibition of both centrally and peripherally induced gastrin release from the antrum

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Palmitate Utilization in Obese-Hyperglycemic Mice

In vitro studies of epididymal adipose tissue and liver

By

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Abstract

Hellman B and S Westman *Palmitate utilization in obese hyperglycemic mice In vitro studies of epididymal adipose tissue and liver* Acta physiol scand. 1964 *61* 65—72 — The in vitro utilization of C_1 palmitate was studied in epididymal adipose tissue and liver slices from obese hyperglycemic mice and their lean littermates Starvation and o addition of glucose to the incubation medium had less effect on the palmitate metabolism of the epididymal fat pad in the obese mice than in the lean controls The presence of glucose thus resulted in a significantly lower recovery of radioactive carbon dioxide and tissue fatty acids in the controls no such effect being noted in the obese mice In both types of mice starvation decreased the incorporation of palmitate carbon in the lipid fraction of adipose tissue incubated in a glucose-containing medium. Fatty acid release from the isolated epididymal fat pad was also less affected in the obese mice than in the lean littermates by starvation and addition of glucose The incorporation of the substrate palmitate into hepatic tissue lipids did not differ significantly between the two types of mice when tested in the presence of glucose In this case starvation resulted in reduced amounts of radioactivity in carbon dioxide and insoluble residue only for the liver slices taken from the lean mice The results are discussed with regard to previous metabolic findings in the obese hyperglycemic syndrome

The central position of the adipose tissue in carbohydrate and lipid metabolism is now well established and much of our effort to elucidate the metabolic anomalies associated with the obese hyperglycemic syndrome has therefore been confined to the fat cells (Hellman *et al* 1962 a b 1963 Westman and Hellman 1962 1963) The lower number of fat cells per unit volume as well as the accumulation of non fat cells in the fat depots of the obese hyperglycemic mice make it difficult to compare directly the metabolism of adipose tissue in normal and obese mice (Hellman *et al* 1962 a 1963) The response of the adipose tissue to different stimuli provides on the other hand a more satisfactory basis for comparison Previous studies showed that the lipogenesis from acetate was

less stimulated by glucose in the obese hyperglycemic mice (Hellman *et al.* 1962b) and only a slight influence on the fatty acid release from the adipose tissue was noted in these animals, when starved (Westman and Hellman 1963). The present communication deals with the *in vitro* utilization of $^{14}C_1$ palmitate in tissues from starved and fed obese hyperglycemic mice and their lean litter mates. The possibility of a defective cell responsiveness being a more general metabolic feature in the obese hyperglycemic syndrome was considered by performing these studies on both the epididymal fat pad and non adipose tissue (liver) with and without glucose added to the incubation medium.

Material and methods

About 8 months old obese hyperglycemic mice from the *R. B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A.* and their lean siblings were used. Eleven obese mice (AO mice) and 12 non-obese animals (AN mice) were allowed free access to food (caloric composition: carbohydrate 54 %, fat 16 % and protein 30 %), while 12 obese mice (AOS mice) and 11 lean controls (ANS mice) were given only water for 48 hours prior to death. All animals were killed at the same time of the day by a blow on the neck and decapitation. The epididymal fat pads were carefully dissected, weighed on a torsion balance and the most distal parts (about 70 mg) taken for incubation. In addition the liver was weighed and thin slices (about 70 mg) prepared for incubation. Macroscopically the livers of the AO mice exhibited an obvious fatty infiltration.

Epididymal adipose tissue was incubated for 3 hours at 37° C under oxygen in 0.5 ml of a modified Krebs Ringer phosphate buffer (calcium omitted) either in the presence or absence of 0.1 % glucose. The medium contained $^{14}C_1$ palmitate (potassium salt from *The Radiochemical Centre, Amersham, England*) and carrier (total concentration 2 mM corresponding to 5 μC/ml) in substitution for sodium chloride and 5 % human serum albumin with a content of approximately 1.6 mmole fatty acids per mmole albumin. All liver slices were incubated in the glucose containing medium, the procedure being identical with that for the adipose tissue.

The respiratory CO_2 was absorbed in NaOH and measured for radioactivity as according to Miller and Hastings (1949). Tissue lipids were extracted with chloroform methanol (2 : 1) and washed (*cf.* Folch *et al.* 1957). In the two phase system thus obtained the triglycerides and fatty acids remain in the lower chloroform phase. Free fatty acids were separated from neutral fat by the method of Borgström (1952) and parts of the two fractions mounted on lens paper-covered planchets (cf. Entenman *et al.* 1949). The insoluble residue left after chloroform methanol extraction and dissolved in 0.1 N NaOH for 24 hours at 20° C and the upper phase were measured for radioactivity. The release or uptake of free fatty acids during the incubation was determined with the modification of Dole's (1956) method as described by Trout *et al.* (1960).

Results

The body weights as well as the weights of the epididymal fat pads and the livers are given in Table I. The results obtained when the epididymal adipose tissue was incubated with $^{14}C_1$ palmitate in media with and without glucose are summarized in Table II. The addition of glucose resulted in a significantly lower oxidation of exogenous palmitate in the AN mice ($t = 4.31$, $P < 0.001$)

Table I. The body weight (g) and the weights (w_w) of the two epididymal fat pads and the liver in fed and starved lean and obese hyperglycemic mice. Mean values ± S.E.M.

Animal	Body weight (g)	Epididymal adipose tissue (mg wet wt)	Liver (mg wet wt)
AN	28.8 ± 1.0	547 ± 53	1,475 ± 67
ANS	24.3 ± 0.5	306 ± 53	1,332 ± 47
AO	56.0 ± 1.4	1,283 ± 149	3,106 ± 146
AOS	46.5 ± 1.2	897 ± 127	2,366 ± 136

Table II. mμmoles palmitate converted into carbon dioxide and different fractions of the epididymal adipose tissue. The results are expressed per 100 mg wet weight. Mean values ± S.E.M.

Animal	Glucose	mμmoles palmitate utilized per 100 mg adipose tissue (wet wt) CO	Upper phase	Neutral fat	Fatty acids	Insoluble residue
AN	−	9.1 ± 1.0	3.9 ± 1.2	150.3 ± 38.5	119.4 ± 15.7	4.4 ± 0.8
AN	+	4.5 ± 0.3	1.3 ± 0.2	40[illegible].8 ± 48.7	49.8 ± 9.6	4.7 ± 0.7
ANS	−	8.5 ± 1.7	8.8 ± 1.1	7[illegible]6 ± 21.9	409.7 ± 27.5	7.5 ± 0.9
ANS	+	8.9 ± 1.8	5.9 ± 0.3	199.2 ± 64.5	312.7 ± 48.7	8.9 ± 1.8
AO	−	17.7 ± 2.8	4.6 ± 0.6	132.7 ± 18.3	61.6 ± 9.1	6.1 ± 1.1
AO	+	12.4 ± 1.3	4.3 ± 0.8	215.8 ± 14.3	55.7 ± 4.5	4.9 ± 0.6
AOS	−	21.4 ± 4.4	4.5 ± 1.0	131.0 ± 16.2	149.8 ± 25.8	8.4 ± 1.8
AOS	+	15.7 ± 2.2	3.5 ± 0.6	153.0 ± 21.1	103.6 ± 16.2	6.0 ± 0.6

but no differences were noted in the ANS mice or in the obese mice. Starvation increased the formation of $^{14}CO_2$ only in the lean mice for the glucose containing medium ($t = 2.57$, $P < 0.02$). After withdrawal of food the lean mice exhibited a higher incorporation of radioactivity in the upper phase for both media, no significant effect of this kind being noted for the obese animals. Addition of glucose increased the incorporation of radioactivity into neutral fat by as much as 170% in the AN mice ($t = 4.05$, $P < 0.001$) and by only about 40% in the AO mice ($t = 2.70$, $P < 0.02$). In the glucose-containing medium starvation resulted in a lower recovery of palmitate carbon from the adipose tissue lipids of both types of mice. In the absence of glucose the nutritional state did not significantly affect the radioactivity of this fraction in either of the two types of mice. Only the AN mice showed a significantly lower radioactivity for the free fatty acids after addition of glucose ($t = 3.78$, $P \approx 0.001$). Regardless of the medium used, starvation increased the amount of radioactivity recovered in the

Table III The release into (+) or the uptake from (−) the medium of free fatty acids (FFA) by the epididymal adipose tissue expressed as μEq fatty acids per 100 mg wet weight and 3 hours incubation Mean values ± S E M

Animal	Glucos	μEq FFA/ 100mg wet wt
AN	−	+0.11 ± 0.16
AN	+	−0.38 ± 0.12
ANS	−	+1.43 ± 0.16
ANS	+	+0.93 ± 0.15
AO	−	−0.10 ± 0.28
AO	+	−0.06 ± 0.08
AOS	−	+0.49 ± 0.10
AOS	+	+0.10 ± 0.12

Table IV mμmoles palmitate converted into carbon dioxide and different fractions of the liver slices The results are expressed per 100 mg wet weight Mean values ± S E M

	mμmoles palmitate utilized per 100 mg liver (wet wt)				
Animal	CO	Upper phase	Neutral fat	Fatty acids	Insoluble residue
AN	12.9 ± 0.5	3.9 ± 0.4	185.3 ± 16.2	61.2 ± 8.7	10.3 ± 1.3
ANS	9.0 ± 0.8	3.6 ± 0.5	55.9 ± 7.6	73.1 ± 7.0	5.3 ± 1.0
AO	9.0 ± 0.8	4.2 ± 0.8	142.9 ± 20.0	65.8 ± 5.8	7.2 ± 0.9
AOS	9.9 ± 1.2	4.1 ± 0.7	73.2 ± 7.8	81.6 ± 6.7	6.2 ± 0.7

free fatty acids for both types of mice The radioactivity of the insoluble residue was not affected by the availability of glucose either in the obese mice or in the lean controls

If expressed per unit fat pad weight all obese mice exhibited a higher oxidation of the substrate palmitate than the lean mice When calculated in this way the amounts of radioactivity recovered in the tissue lipids were on the other hand lower in the AO mice than in the lean controls for the adipose tissue incubated with glucose

The release or uptake of free fatty acids from the isolated epididymal adipose tissue is shown in Table III The fed lean mice showed an uptake of free fatty acids in the presence of glucose Withdrawal of food resulted however in a fatty release which was more pronounced after omission of glucose The fatty acid release from the epididymal fat pad was less affected by starvation and addition of glucose in the obese mice than in the lean littermates The starved

obese mice displayed a fatty acid release only if the incubation was performed without glucose

The results obtained when the liver slices were incubated with $^{14}C_1$ palmitate in a glucose containing medium is presented in Table IV In the AN mice starvation resulted in a significantly lower oxidation of the substrate palmitate ($t = 3.56$ $P < 0.01$) a considerable reduction of incorporation into neutral fat ($t = 7.04$ $P < 0.001$) as well as lower values for the radioactivity in the insoluble residue ($t = 3.37$ $P < 0.01$) Also for the AO mice withdrawal of food was reflected in a reduction of the radioactivity in the neutral fat ($t = 3.36$ $P < 0.01$) A comparison between the two types of mice revealed a significant depression of the $^{14}CO_2$ formation in the fed obese hyperglycemic mice ($t = 3.53$ $P < 0.01$) Neither the liver slices from the lean nor obese mice showed any fatty acid release irrespective of the nutritional state of the animals

Discussion

The isolated adipose tissue not only incorporates fatty acids into the neutral or glyceride component but also oxidizes fatty acids to carbon dioxide (Shapiro *et al* 1957 Milstein *et al* 1959) Both processes are markedly influenced by the nutritional state of the animals and the availability of glucose (*cf* Raben and Hollenberg 1959) The present experiments corroborate these findings the epididymal adipose tissue from the fed lean mice exhibited a significantly lower oxidation and a markedly increased incorporation of exogenous palmitate carbon atoms into neutral fat after addition of glucose A sparing effect of glucose on the *in vitro* oxidation of fatty acids has been described also in the adipose tissue from the rat (Bally *et al* 1960) The glycerophosphate required for synthesis of triglycerides from fatty acids is derived from glucose The enhancement of glucose on this esterification process was reflected in the adipose tissue of the lean mice by the greater radioactivity in the neutral fat as well as by the lower recovery of the palmitate carbon in the free fatty acid fraction After starvation of the lean mice a smaller proportion of the assimilated radioactive fatty acid appeared esterified while there were greater amounts of radioactive carbon dioxide A proportionately greater oxidation of fatty acids in fasted animals than in refed animals has previously been reported by Shapiro *et al* (1957) and attributed to the larger free fatty acid pool obtained by starvation

It emerged from our previous studies that the stimulation of the adipose tissue lipogenesis from acetate obtained by adding glucose to the incubation fluid was less pronouced in the obese hyperglycemic syndrome (Hellman *et al* 1962 b) In the present investigation starvation and/or addition of glucose had less effect on the palmitate metabolism of the epididymal fat pad in the obese mice than in their lean controls The presence of glucose resulted for example in a significantly lower recovery of radioactive carbon dioxide and tissue fatty acids in the controls no such effect being noted in the obese mice After starva

tion the amounts of radioactivity in the upper phase and insoluble residue were significantly increased in the epididymal adipose tissue taken from lean mice but not from obese animals The absence of a marked response to fasting or addition of glucose apparently represents a more general metabolic feature for the epididymal fat pad in the obese hyperglycemic syndrome Up to now it is difficult to assess to what extent this non responsiveness of the intermediary adipose tissue metabolism may reflect an impairment of the glucose metabolism Christophe *et al* (1961) reported the glucose utilization to be depressed in the isolated epididymal adipose tissue of obese hyperglycemic animals either when the metabolic data were expressed per unit nitrogen or wet weight

An impaired mobilization of free fatty acids has been suggested to be a metabolic defect of significance for the etiology of the type of adipositas represented by the obese hyperglycemic syndrome (Marshall and Engel 1960 Leboeuf *et al* 1961) In our previous studies a more pronounced effect of fasting was also noted in the lean littermates when the incubation of the epididymal fat pad was performed in a medium containing glucose and only those fatty acids bound to the unextracted albumin (Westman and Hellman 1963) In the present experiments it was proved with a higher initial concentration of fatty acids in the incubation medium that both glucose and starvation have some influence on the fatty acid release from the isolated epididymal fat pad of obese hyperglycemic mice but to a much less extent than noted for the lean littermates

In a comparison of the lipogenic activity of the epididymal adipose tissue from normal and obese hyperglycemic mice Hellman *et al* (1962 a) demonstrated the different number of fat cells per unit weight made it necessary to express the metabolic data directly per fat cell The relative nitrogen content of the epididymal fat pad remained unchanged in the obese mice in spite of the marked reduction in the number of fat cells per unit weight The more recent observation of a greatly increased mast cell content in the adipose tissue from obese hyperglycemic mice stresses the importance of taking into account the number of fat cells when comparing the adipose tissue metabolism in normal and obese hyperglycemic mice (Hellman *et al* 1963) The choice of parameter for the metabolic data seemed however to be of minor importance in the present experiment where the effect of glucose and a short term starvation was tested using the same type of mice as controls Even if the adequate comparison between the adipose tissue metabolism of the two types of mice was confined to the reactions to glucose and starvation the previous observation of 2—3 times more fat cells per unit tissue weight in the adult lean mice (Hellman *et al* 1962 d) gives some idea how the values listed in the Tables will change when calculated per fat cell (*cf* Hellman *et al* 1962 a)

In previous analyses of the liver metabolism in obese hyperglycemic mice ^{14}C-glucose labelled in different positions has been used as a radioactive precursor (Hellman *et al* 1961 1962 c) While no significant changes from the normal level were noted for the *in vitro* utilization of glucose in liver slices devoid

of macroscopical fatty infiltration the presence of a marked liver fattening was found to be associated with a somewhat depressed ability to metabolize glucose also when the results were expressed in terms of fat free dry weight In view of the accelerated lipogenesis from acetate described by Mayer *et al* (1955) as characteristic for liver slices from obese hyperglycemic mice it is worthy of note that the synthesis of neutral fat from the substrate glucose was slightly depressed in our experiments (Hellman *et al* 1962 c) It should however be noted that the observations of Mayer *et al* (1955) of a greater hepatic lipogenesis in the obese mice refer to experiments performed without glucose in the incubation medium In the present experiments no significant differences were obtained for the palmitate incorporation into hepatic tissue lipids between the obese hyper glycemic mice and their lean littermates The finding that starvation resulted in significantly reduced amounts of radioactivity in the CO and insoluble residue for the lean but not the obese mice is probably another expression of the disturbed metabolism in the obese hyperglycemic syndrome In an assess ment of these results the possibility of a differential dilution of the labelled precursor with endogenous fatty acids in the two types of starved mice should be considered Zomzely and Mayer (1959) found that the dilution of labelled acetate was of the same order of magnitude when the animals were allowed free access to food but that the endogenous acetate pool was proportionately greater in starved obese hyperglycemic mice

A release of considerable amounts of free fatty acids has previously been encountered for rat liver slices incubated in different media and the conclusion was drawn that in addition to adipose tissue the liver represents a source of these compounds (*cf* Hamosh and Shapiro 1961) The absence of any release of fatty acids from the liver slices taken from the lean and obese mice may be explained by the presence of glucose and the relatively high concentration of fatty acids in the medium

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The Measurement of Input Impedance and Apparent Phase Velocity in the Human Aorta

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Abstract

Gabe I T J Karnell I G Porje and B Rudewald *The measurement of input impedance and apparent phase velocity in the human aorta* Acta physiol scand 1964 61 73—84 Measurement of the input impedance in the aorta in 3 patients with rheumatic mitral disease is described The aortic flow was measured by recording the pressure gradient along the ascending aorta with a differential manometer through a double lumen catheter a separate strain gauge on one side of the system enabled the arterial pressure to be recorded simultaneously Records were made before and during the administration of i v nor adrenaline Computation of blood flow apparent phase velocity and impedance was performed on a digital computer The moduli of aortic input impedance decreased with frequency over the first few harmonics both before and during nor adrenaline but were higher during infusion The pattern of impedances tended to suggest that some reflection was reaching the ascending aorta this was also suggested by the high values obtained for apparent phase velocity at low frequencies

The calculation of peripheral vascular resistance from mean cardiac output and mean aortic blood flow is a common and simple way of assessing the load which faces the left ventricle However the load consists of a branched elastic arterial system and both aortic blood flow and pressure have large oscillatory components in these circumstances a description of the load in terms of resistance alone is inadequate and a more general approach is clearly desirable One such approach is the calculation of the aortic input impedance (McDonald and Taylor 1959 McDonald 1960)

The concept of impedance is widely used in electric alternating-current theory and is also usefully employed in mechanics and acoustics The calculation of impedance can only be carried out if the variations of pressure

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and flow are sinusoidal or periodic Although the changes of pressure and flow in the arterial system are certainly not sinusoidal they are almost periodic and both pressure and flow waveforms may be expressed as Fourier Series that is, each may be shown to consist of a mean term and the sum of a series of harmonics The ratio of the mean pressure to the mean flow is the conventionally calculated peripheral vascular resistance The complex ratio of each harmonic of pressure to the corresponding harmonic of flow is the impedance at the particular frequency involved

The principal object of this communication is to present measurements of the input impedance of the aorta of three patients before and during the infusion of noradrenaline Essential to such calculations are measurements of aortic blood pressure and flow waveforms The recording of blood pressure presents no great problems, instantaneous blood flow is still, however measured with some difficulty Electromagnetic and ultrasonic flow meters cannot be used without a great deal of difficulty on intact man and the only practicable method of measuring aortic blood flow at present is by a pressure gradient technique (McDonald 1955 Womersley 1955 Fry 1956 Porje and Rudewald 1957, 1961)

Methods

Apparatus The differential manometer and double lumen catheter have been described previously by Porjé and Rudewald (1961) The manometer differed from previous models in that the volume displacement was smaller In order to measure the arterial pressure and the pressure difference simultaneously a Statham P23Gb strain gauge was mounted on one side of the differential manometer The system was so connected that the single sided arterial pressure was recorded from the opening at the tip of the catheter The manometer system was filled with recently boiled distilled water containing 0 5 % benzalkonium chloride Records were made on an Elema 4 Channel Direct Writing Recorder No electrical or additional hydraulic damping was employed The frequency response of the whole system was tested with a sinusoidal pressure generator (Ball and Gabe 1963) and both the differential pressure and single sided pressure records showed a resonance at 31 c/s the amplitudes at resonance relative to the amplitude at 0 01 c/s were 4 7 and 4 5 respectively The amplitude and phase distortion was measured and this data enabled small corrections to be made during computations

Technique At the time of study the patients investigated were undergoing diagnostic cardiac catheterization and angiocardiography for rheumatic mitral stenosis and incompetence All the patients were in sinus rhythm After pressures and samples had been taken through an intravenous cardiac catheter the double lumen catheter was introduced percutaneously into the femoral artery through a modified Gidlund cannula (Rudewald 1962) The catheter was advanced under fluoroscopic control to the ascending aorta so that the catheter opening at the tip was just above the aortic valve Biplane radiograms were taken to record the position of the catheter Records of the differential pressure and aortic pressure were made and during this time the cardiac output was measured by the Fick method A slow intravenous infusion of nor adrenaline was then begun (at approximately 0 5 μg/kg/min) and pressure records once again were made during this period the cardiac output was also once more estimated *Finally* an angiocardiogram was performed and from this the mean diameter of the ascending

aorta could be calculated as well as the effective distance between the pressure sampling points The effective distance was between 6 and 7 cm in the 3 cases

Computations Application of the Navier Stokes Equation to the flow of a Newtonian fluid passing periodically along a rigid cylindrical tube makes it possible to determine the flow if the pressure gradient along the tube is known. We may express the pressure gradient in terms of a Fourier Series

$$A_0 + \sum_{n=1}^{\infty}(A_n \cos n\omega t + B_n \sin n\omega t)$$

The appropriate expression for flow due to Womersley (1955 1957) is

$$F_n = \frac{\pi R^4}{n\omega\rho}\left[(A_n C_n + B_n D_n)\sin n\omega t + (A_n D_n - B_n C_n)\cos n\omega t\right] \qquad (1)$$

where F_n = the volume flow of the nth harmonic
R = radius of the tube
ρ = density of fluid (taken as 1 06 g cm^{-3} for blood)
ω = $2\pi \times$ fundamental frequency

C and D are the real and imaginary parts of $1 - \dfrac{2J_1(\alpha i^{3/2})}{\alpha i^{3/2} J_0(\alpha i^{3/2})}$ where α is the non-dimensional parameter $R\sqrt{\dfrac{n\omega}{\nu}}$, ν being the kinematic viscosity (taken as 0 04 cm^2 sec^{-1} for blood)

The calculation of flow using this equation may most conveniently be carried out by a digital computer The heights of the differential pressure and pressure records were measured at 10 msec intervals from a convenient common baseline beginning and ending at successive R waves This data together with information on calibrations radius of aorta density of blood value of α for the fundamental frequency and the corrections to be applied for amplitude and phase distortion of the transducer and recording system was punched on to 5 hole paper tape A suitable programme for the computation of the Fourier coefficients of differential pressure and aortic pressure and for the derivation of the oscillatory components of blood flow was written for the University of London Ferranti Mercury computer Included in this programme was the computation of input impedance of the aorta from the complex ratio of pressure to flow at each harmonic The total time taken for the computations from a single pulse was between 2 and 3 min

It was decided to carry out the computations to ten harmonics It is to be expected that the accuracy of the values obtained for impedance will decrease with frequency The magnitude of the harmonics of pressure and flow diminishes with frequency and the signal to-noise ratio consequently falls In addition, the measurement of the pressure difference between two points a discrete distance apart is a substitute for the ideally required pressure gradient at a point Estimated flow from the pressure difference waveform is for a point midway between the two measuring sites The assumption is made in calculating impedance that the computed flow at this point differs little from the flow pattern at the entrance of the aortic segment (where the single sided aortic pressure is measured) In the low frequency range (up to perhaps 5 c/s) the error which is largely one of phase should be small but at higher frequencies the error will certainly be greater Nevertheless it was thought that it would be of interest to give the results to ten harmonics because precise evaluation of accuracy is not possible with present methods

The pressure gradient along the aorta is produced by two main factors first by the acceleration and deceleration of blood and secondly as a result of viscous forces. In

Table I

Patient	Before noradrenaline			During noradrenaline		
	Mean BP (mm Hg)	Cardiac output (l/min)	Peripheral resistance dyne sec cm^{-5}	Mean BP (mm Hg)	Cardiac output (l/min)	Peripheral resistance dyne sec cm^{-5}
B S age 37	83	5 8	1 140	114	6 5	1 400
S N age 39	94	6 2	1 210	126	5 4	1 870
I S age 37	97	4 9	1 580	138	5 9	1 870

the ascending aorta of man the inertial factor is dominant and we may see the effect on equation 1 of assuming that the fluid has zero viscosity by making α infinite It may be shown that the real and imaginary parts of $1 - \frac{2 J_1(\alpha_n i^{3/2})}{\alpha_n i^{3/2} J_0(\alpha_n i^{3/2})}$ then become unity and zero respectively and that

$$F_n = \frac{\pi R^2}{n\omega\rho}(A_n \sin n\omega t + B_n \cos n\omega t) \tag{2}$$

Thus the oscillatory components of flow in this case may be derived by simple integration of the waveform of pressure gradient a method that has been used in the human aorta by Porjé and Rudewald (1957 1961) In order to compare the results obtained by the exact solution of the Navier Stokes Equation with those obtained by assuming an ideal fluid the computations were repeated with the instruction that α for the fundamental frequency was to be taken as 10 000 (and 10 000$\sqrt{n}$ for higher harmonics) a value high enough to ensure that equation 2 would be computed with negligible error

Since the absolute pressure at one side hole of the double lumen catheter was known as well as the pressure difference between the two side holes calculations of the apparent phase velocity at each harmonic could easily be derived from the results of the two Fourier analyses. If $\Delta\Phi$ radians is the phase difference of the absolute pressure wave forms per cm length of aorta at a frequency f c/s then the apparent phase velocity is $2\pi f/\Delta\Phi$ cm sec^{-1}

Results

The calculated aortic input impedances derived from single pulse periods before and during the i v administration of noradrenaline are shown in Fig 1 2 and 3 for patients B S S N and I S respectively It will be seen that in each case the moduli of the first few harmonics increased when noradrenaline was given as did the peripheral resistance (Table I) The fact that the moduli of the early harmonics decreased with frequency (with the exception of I S before noradrenaline) suggests that the input impedance of the aorta at these frequencies is capacitative and this is supported by the negative phase angles indicating that flow is leading pressure in phase With increasing frequency the phase angles become less negative The accuracy of the values obtained for the moduli and phase angles will necessarily fall with increase of frequency

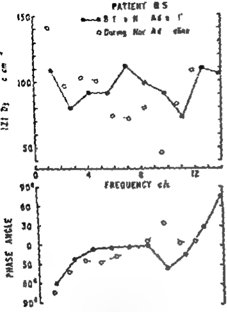


Fig 1

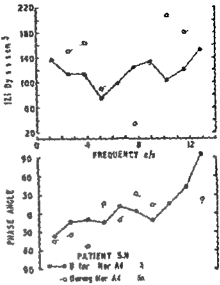


Fig 2

Fig 1 (Left.) Aortic input impedance as a function of frequency before and during intravenous noradrenaline Patient B S

Fig 2 (Right) Aortic input impedance as a function of frequency before and during intravenous noradrenaline Patient S N

(see above) It will be seen in Fig 2 and 3 that the phase angles of 3 harmonics exceeded ± 90 and these angles must certainly be in error Fig 4 shows the mean values of impedances obtained by averaging results from 3 pulse periods from each patient first before and then during noradrenaline Frequencies were grouped into class intervals of 1 c/s

If the assumption is made that the viscosity of blood is zero (or that α is infinite) the error which will then be made in the calculation of the flow harmonics and consequently in the values for impedance may be deduced theoretically It can be shown that

$$\frac{F_\infty}{F} = \frac{1}{M_{10}} \underline{|-\epsilon_{10}}$$

$$\simeq \frac{\alpha^2}{\alpha^2 - \alpha\sqrt{2} + 1} \underline{|-\epsilon_{10}} \quad (\alpha > 10)$$

where F_∞ and F are the derived flows at infinite α and appropriate α respectively and M_{10} and ϵ_{10} are functions of α and are defined by Womersley (1955) Fig 5 shows the ratio F_∞/F plotted against α The values of α for the fundamentals in the three patients ranged from 16 0—24 2 It will be seen that the

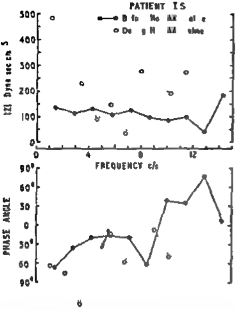


Fig 3

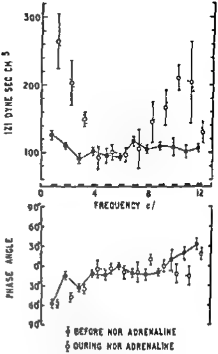


Fig 4

Fig 3 (Left) Aortic input impedance as a function of frequency before and during intravenous nor adrenaline Patient I S

Fig 4 (Right) Mean values for aortic input impedance The values derived from the analysis of three pulse periods from each patient were averaged first before and then during the administration of nor adrenaline Frequencies were grouped into class intervals of 1 c s The vertical bars indicate the standard error of the mean

error to be expected in the estimation of the modulus of flow when viscous effects are neglected is about 10 % if α is 16 the phase angle is then in error by -5 These errors diminish at higher values of α The flow harmonics calculated using a value of 10 000 for the α of the fundamental (and $10\,000\sqrt{n}$ for the higher harmonics) were entirely compatible with these predictions a fact which served to check the correctness of part of the programme and its execution

An example of the effect of assuming zero viscosity on the synthesised wave form of aortic blood flow is shown in Fig 6 The continuous curves in Fig 6 are the same waveforms of oscillatory aortic blood flow derived using correct values of α The value of α for the fundamental in this case was 19 1 Superimposed on the continuous curve in Fig 6(a) is the waveform of flow calculated using a value of 10 000 for the α of the fundamental this flow curve is also shown in Fig 6(b) but its amplitude has been reduced uniformly by 8 % For the purpose of comparison each curve has been drawn about its individual

Fig 5 F_{∞}/F as a function of α. F_{∞} is the flow which will be calculated from a sinusoidal pressure gradient if the effects of viscosity are neglected (or α is infinite). F_{α} is the flow derived from the solution to the Navier Stokes Equation applied to a viscous fluid in a long cylindrical tube (Equation 1)

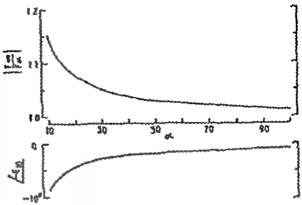
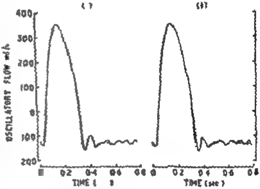


Fig 6 Synthesised waveforms of aortic blood flow in patient B N during the administration of noradrenaline. The first ten harmonics were used. The continuous curves are the same flow wave forms derived by Equation 1. The dotted curve superimposed on this in Figure 6 (a) is the waveform of flow obtained when values for α were taken to be $\geqslant$ 10 000 that is the blood was assumed to be without viscosity. The dotted curve in 6 (a) has been redrawn in 6 (b) but its amplitude has been reduced in scale by 8 %. To facilitate comparison each curve has been drawn about its individual mean and the mean forward flow has not been included in the scale. The value of α for the fundamental in this case was 19 1



mean and the average forward flow has not been added. It is evident that the error produced when viscosity is neglected is much reduced by the change of scale even though the attempt at correction is crude: all the harmonics are in effect reduced by 8 % in amplitude while the phase relationships are unaffected. The use of a simple factor for correcting the fluid inductance of an ideal fluid was first suggested by Fry (1959)

In Fig 7 are shown the apparent phase velocities derived from the analysis of single pulse periods in each patient before and during noradrenaline. Four of the values calculated were negative and these have been omitted from the diagram. In each case the apparent phase velocities of the early harmonics were higher while noradrenaline was being given than before. The mean values obtained for apparent phase velocity in the 3 patients are given collectively in Fig 8. For the preparation of this diagram results from the analysis of 3 separate pulse periods recorded from each patient were pooled, first before and then during noradrenaline. The frequencies were grouped into class inter

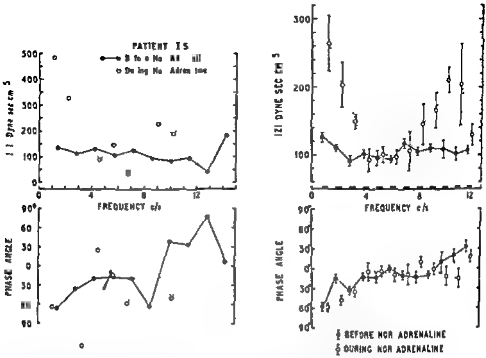


Fig 3 Fig 4

Fig 3 (Left) Aortic input impedance as a function of frequency before and during intravenous nor adrenaline Patient I S

Fig 4 (Right) Mean values for aortic input impedance The values derived from the analysis of three pulse periods from each patient were averaged first before and then during the administration of nor adrenaline Frequencies were grouped into class intervals of 1 c/s The vertical bars indicate the standard error of the mean

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only be the same as the value obtained for peripheral resistance (derived from the ratio of mean arterial pressure to mean blood flow) if the system is linear through the origin The situation is similar to the problem of resistance in a non linear electronic device For example peripheral resistance may be considered as analogous to the equivalent d c resistance of a thermionic valve — the ratio of total anode potential to total anode current This in general differs from the a c anode resistance which is the ratio of a small change of anode potential to the resulting small change of anode current at a particular working point It is unlikely that the non linearities of the vascular system are so great that the peripheral resistance will be of a different order of magnitude from the resistance at zero frequency at the mean arterial pressure but if it is decided to express impedance non-dimensionally as Z/R (Patel *et al* 1963) where Z is the modulus of input impedance and R is the peripheral resistance the distinction should be borne in mind

It is of interest to consider whether the results suggest the presence of reflections in the ascending aorta It may be useful to recall the phenomena which we should expect to find in a highly simplified model system when reflections arise (Taylor 1957) If a rubber tube filled with a viscous fluid is occluded completely at one end and connected at the other to a pump generating sinusoidal fluid flow the input impedance may be greatly affected by reflections As frequency increases from zero the input impedance will be capacitative and will fall until a minimum is reached when the tube is one quarter of a wavelength long The phase angle will then be zero Further increase in frequency results in a rise in input impedance with a positive phase angle a maximum is reached when the tube is half a wavelength long In brief the input of such a system exhibits a series of minima and maxima at multiples of the frequency at which the first minimum appeared The magnitude of the variations will depend mainly on the percentage reflection at the distal occluded end and on the viscous losses along the tube

The branched tapering elastic arterial system is much more formidably complex than the closed rubber tube model but we may reasonably expect even in such a system that systematic variations of input impedance with frequency will occur if significant reflection takes place Before nor adrenaline changes in impedance with frequency are small and must be interpreted with caution (Zeros in Fig 1 2 and 4 are suppressed and changes may in consequence appear to be magnified) In Fig 1 2 and 3 we see that before nor adrenaline there is a minimum at 2 8 c/s in Patient B at 5 1 c s in patient S and in patient I there is little change in the moduli to be observed In the first two patients maxima are to be seen at 7 0 and 9 8 c s in a simple idealised reflecting system we should expect from the frequency of the first minima that the first maxima should be at 5 6 and 10 2 c/s

The apparent phase velocity in the simple tube system is also affected by reflections being initially higher at very low frequencies In 1946 Porje recorded

in man the pulse waveform in the subclavian artery and in the abdominal aorta and made calculations of the apparent phase velocities of the first three harmonics, the value obtained for the apparent phase velocity of the fundamental and frequently also of the second harmonic was considerably higher than the foot to foot velocity These findings were interpreted as being the result of reflections Inspection of Fig 7 and 8, which show apparent phase velocity in the ascending aorta as a function of frequency, reveals high values again at the low frequency end and supports the suggestion that some reflection reaches the ascending aorta

In 1961 Caro and McDonald studied the relation of pulsatile pressure and flow in the pulmonary vascular bed of the rabbit by superimposing a sinusoidal flow generated by a pump on mean steady flow By this method they were able to vary frequency continuously and were not committed to studying impedance at integral multiples of the pulse frequency They found that the input impedance was high at low frequencies and fell to a minimum at 3—4 c/s They pointed out that the fact that this was approximately the normal pulse frequency for the rabbit possibly had relevance in minimising the oscillatory load on the right ventricle Such evidence as has been obtained in this present small series in man suggests that for the ascending aorta the first minimum of input impedance is at a frequency above pulse frequency If no change takes place in the periphery an increase in heart rate would tend to reduce the impedance for the fundamental and the second harmonic

The moduli of impedance for the first few harmonics are greater during noradrenaline infusion than before Such an effect is to be expected when there is increased vasoconstriction peripherally The rubber tube model considered above was completely occluded at one end its resistance at zero frequency would be infinite If the end were constricted but not occluded the resistance at zero frequency would be finite and the input impedance would be lower as frequency began to increase than in the case of total occlusion However, a similar effect could be produced by changing the compliance of the tube There is no doubt from the rise in peripheral resistance in the three patients when noradrenaline was given that there was then increased peripheral vasoconstriction and that this could account for the higher impedance at low frequencies A concomitant increase in the tone of the large arteries would also tend to increase impedance although probably this action would be relatively less important than the action on the arterioles

The accuracy with which aortic input impedance can be measured is dependent mainly on the accuracy of the estimations of instantaneous blood flow Flow measurement by the pressure gradient technique requires the fulfilment of a series of assumptions well stated by McDonald and Taylor (1959) In brief it must be assumed that the tube — in this case the ascending aorta — is long cylindrical and rigid that the flow of blood is laminar and that the blood has Newtonion viscous properties None of these restrictive assumptions can be

completely satisfied in the case of the aorta although McDonald and Taylor give reasons for supposing that the errors involved may not be great Because of the high values of α in the ascending aorta it is clear that inertial effects dominate over viscous factors Treating blood as a viscousless liquid does not greatly change the waveform of computed oscillatory flow as shown in Fig 6 and it may be seen from Fig 5 that calculations of input impedance made on this basis would not have altered the pattern shown in Fig 1 2 and 3 at all significantly

The actual calculations in this work were done on a digital computer Certainly this is preferable to manual computation for once the program is working satisfactorily the chances of error are small The most time consuming part of the procedure is the measurement by hand of the heights of the waveforms of pressure and pressure difference at 10 msec intervals The use of an analogue to digital converter would remove the need for this and would provide data tape which could be fed directly to the computer

Other methods of computation are of course available Recognising that viscous forces in the ascending aorta were small Porje and Rudewald (1957) computed aortic blood flow in man from the pressure gradient by numerical integration the use of electrical integration was described by Rudewald (1962) Fry Noble and Mallos (1957) have described an analogue computer for the derivation of flow from the pressure gradient this was further discussed by Fry (1959) and was used by Barnett Greenfield and Fox (1961) The operation of the computer involves certain simplifications in the equations of flow but an attempt is made to allow for the effects of viscosity The reasonable assumption is made that in the ascending aorta the flow is virtually zero at the end of diastole and the control determining the magnitude of the viscous effects in the computation is so adjusted that this boundary condition is fulfilled However this condition might appear to be difficult to satisfy for essentially it is required that the small pressure drop resulting from the mean component of blood flow be accurately measured the pressure gradient associated with all the oscillatory components of flow averaged over an integral number of cycles must necessarily be zero and therefore the contribution made by each of these can give no information on the position of true zero Thus if the flow within the aorta is 5 l/min the diameter of the aorta is 2 5 cm and the coefficient of viscosity is taken as 0 04P it follows from the Hagenbach Poiseuille equation that the mean pressure drop over a distance of 5 cm would be 0 018 cm H_2O Detection of such a small pressure difference along the aorta presents great difficulties It may be noted that corrections should be made if the catheter openings are not in the horizontal plane Since the specific weights of blood and saline differ a pressure difference will be recorded if a vertical distance separates the two catheter openings this amounts to the product of the vertical distance and the difference in the two specific weights A pressure difference of 0 018 cm H_2O may be produced if the vertical distance is only 3 mm

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The Excitation of Thalamic Neurones by Acetylcholine

By

P ANDERSEN[1] and D R CURTIS

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Abstract

ANDERSEN P and D R CURTIS *The excit tion of thalamic neurones by acetyl hol ne* Acta physiol scand 1964 *61* 85—99 — Electrophoretically ad ministered acetylcholine (ACh) excited neurones located within the ventrobasal thalam c complex of cats an sthetised with pentobarbital All ACh sens tive neurones were fired synaptically by volleys in cutaneous ner e fibres thalamic relay neurones which were fired ant dromically from the primary sensory cortex were more sens tive than thalamic interneurones In co npa ison with Renshaw cells the excitatory act on of ACh was of slow on et and pro longed durat on the spontaneous spindle activity of some cells was depressed pr or to excitation Possible explanations of these time d fferences between the excitant of ACh upon thalamic and Renshaw cells are discussed

For many years there has been considerable interest in the possible role of ACh as a synaptic transmitter within the mammalian central nervous system There is evidence that this substance is an excitatory transmitter of the spinal Renshaw cells (Eccles Fatt and Koketsu 1954 Curtis and Eccles 1958b) but until recently the evidence concerning a transmitter function in higher centres has been indirect (Feldberg 1945 1950 1957 Hebb 1957, 1963)

A necessary prerequisite to the identification of a substance as a transmitter acting upon particular neurons is the demonstration that it has the same action upon the postsynaptic membrane as does the transmitter In order to satisfy this requirement it is essential that a method of administration is chosen which permits ready access of the substance to its supposed site of action It is now possible to administer pharmacologically active compounds from multibarrel micropipettes near selected neurones (Curtis 1964) This micro-electrophoretic technique has been used to demonstrate ACh sensitive neurones in the spinal cord (Curtis and Eccles 1958a) brain stem (Curtis and Koizumi 1961, Bradley

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and Wolstencroft 1962 Salmoiraghi and Steiner 1963), lateral geniculate nucleus (Curtis and Davis 1963) and the cerebral cortex (Krnjevic and Phillis 1963b Spehlmann 1963 Crawford and Curtis 1964) The present report concerns the excitation by ACh of cells in the ventrobasal complex of the feline thalamus an important relay nucleus in the somesthetic pathway A subsequent paper will deal with the pharmacology of this action Preliminary reports of this investigation have been published (Curtis and Andersen 1962, Curtis 1963b)

Methods

All experiments were performed upon cats anesthetised with pentobarbital sodium. The temperature of the preparations was maintained at 37 ± 1 C by two heating pads The neurones were located in the region of the left nucleus ventralis posterolateralis (VPL) and nucleus ventralis posteromedialis (VPM) and were identified by the responses which followed electrical stimulation of the contralateral infra orbital median ulnar superficial radial tibial peroneal or sural nerves The infra orbital nerve was stimulated with needle electrodes inserted into the infraorbital foramen and the other nerves were mounted on platinum electrodes in paraffin pools The left cranial vault was removed and the dorsal surface of the thalamus exposed by partial removal of the overlying cerebral cortex, without damaging the thalamo-cortical projection to the post cruciate area Thalamic neurones were located stereotaxically by passing 5 barrel micropipettes in a vertical plane using the co ordinates published by Jasper and Ajmone Marsan (1954) Usually the electrode was passed through a hole in a small pressure plate which was held in position by a second manipulator The head of the animal was fixed in a modified Horsley Clarke type of head frame and both the pelvis and thorax were suspended above the base of the animal frame These manoeuvres overcame many of the difficulties produced by respiratory movement of the cerebral tissue The exposed thalamus and surrounding brain were continuously flushed with mammalian Ringer solution of 37 C Bipolar platinum stimulating electrodes were placed on the surface of the primary somatosensory cortex and were surrounded by cotton wool soaked in paraffin (B P) to prevent drying of the exposed cortex

The 5 barrel micropipettes used in this investigation had a total tip diameter of 4–8 μ the centre barrels were filled with 5 M NaCl and the 4 outer barrels contained aqueous solutions of the substances which were to be tested The dry micropipettes were filled from above with fine polyethylene tubing and filling was completed by centrifugation All pipettes were examined microscopically before and after use Retaining potentials of the order of 0 5 V were applied to the individual drug barrels in order to control the diffusional efflux of active materials In general solutions of 0 1 – 1 M were used and care was taken to fill the micropipettes with solutions of the same molarity when comparing the potency of different excitants Active cations were ejected by an outward or cationic current and anions were ejected electrophoretically by an anionic current. All currents are given as nA (10^{-9} A) and were measured with an accuracy of $\pm$ 0 5 nA When comparing the activities of different substances care was taken to eject them for prolonged periods (up to 3 min) if the potency appeared to be low Furthermore excitants were compared only after several ejections so that each substance gave a maximal response for a particular electrophoretic current This procedure also ensured that responses occurred with a minimal latency (see Curtis and Watkins 1963) The times of cessation of drug action following termination of the ejection were compared after equal firing frequencies had been obtained and when after-diffusion was limited by the use

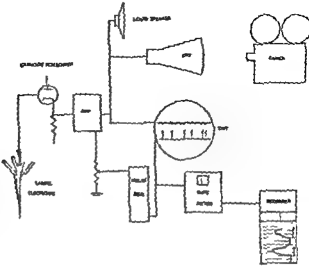

Fig 1 Diagram of the equipment to monitor and record the responses of single thalamic neurones (see text)

of excessive retaining potentials One barrel of the majority of micropipettes contained an 0 2 M solution of DL-homocysteic acid (pH 8 NaOH) The ejection of such an excitant amino acid proved to be of considerable assistance when assessing the excitant or depressant properties of other substances (see Curtis and Davis 1963)

The recording microelectrode was connected by means of a small Ag—AgCl junction to a negative capacitance cathode follower (Fig 1) After suitable amplification (AMP) the spike potentials (negative — positive 0 2—0 5 mV) were displayed upon an oscilloscope (CRT 1) from which photographic records could be made In addition the spikes were used to trigger a pulse generator (PULSE GEN) Both the output pulses of this generator (50 V 100 μsec) and the spike potentials were observed upon a double beam oscilloscope (CRT 2) the triggering level of the generator could then be adjusted with a potentiometer P in order to select only the larger spikes The pulses were counted by means of a rate meter (Nuclear Enterprises NE 5401) which was modified to give time constants of 0 1 0 2 0 5 or 1 sec for the range 0—100 pulses per sec The output of the rate meter was displayed on an ink recorder (Texas Instruments Recti/Riter) upon which side marker pens indicated periods of drug ejection. The response time of this recording system was comparatively slow (see Fig 4) but the continuous display of firing frequency both spontaneous and drug induced was invaluable for determining the potency of the various cholinomimetics and for observing the actions of blocking and potentiating agents Because of the slow paper speed which was used (1 1 2 inches per min) the latencies of onset and offset of drug action could not be measured accurately unless these times exceeded 2—5 sec However shorter intervals were readily measured from photographic records

Tissue Blocking of Micropipettes

A major difficulty was encountered in this investigation which has not been observed to the same extent in previous studies of this nature Once single neurones were located it was common to find that cells having extracellular spike potentials of the order of 0 2—0 5 mV (negative positive) appeared to be relatively insensitive to the excitant action of L-glutamic or DL-homocysteic acids Firing of these cells (hereafter referred to as the major neurone) was delayed for 3—60 sec, and was usually preceded by high fre

quency firing of other cells which had smaller spike potentials and which were presumably further from the orifice of the recording electrode than was the major cell under observation When the amino acid induced firing of the major cell its time course was very similar to that observed when spinal interneurones were activated by these particular amino acids (see Curtis and Watkins 1963) On rare occasions even DL-homocysteic acid failed to fire neurones although other nearby cells became so depolarized by the amount of amino acid ejected that the spike mechanisms became inactivated In many cases it proved possible to rectify this apparent inability to excite neurones by withdrawing the micropipette 5–20 μ from the site of recording but the resultant reduction in the amplitude of the spike occasionally led to difficulty in identifying the particular neurone amongst other nearby cells

These observations suggested that there was a barrier which hindered the passage of ejected ions to the cell from which large spike potential could be recorded Invariably there was an associated increase in the electrical resistance of the various drug containing barrels and the resistance of the central recording electrode often doubled from an original value of 4–8 MΩ Occasionally difficulty was experienced in passing electrophoretic currents greater than 20 nA particularly in the cationic direction Furthermore the electrical coupling between the various barrels increased and this raised the possibility that the potential set up across this resistance by the passage of current through any one barrel could interfere with the ejection or the control of the diffusional efflux of substances in the other barrels Confirmation of the presence of such a mechanical barrier was obtained when the tips of the micropipettes were examined microscopically using a water immersion objective The tips of the 5 barrel micropipettes were found to be surrounded by a roughly spherical outgrowth of diameter 10–30 μ portion of which presumably partially plugged the orifices of individual barrels After storage or rinsing in water this was converted to a cap of material of high refractive index which tapered off along the shaft for a distance of 5–80 μ The thickness at the electrode tip was of the order of 1–3 μ and the caps often displayed a layered appearance as if the electrode successively accumulated cellular fragments or myelin in its passage through cerebral tissue It was not possible to remove these structures by a high pressure water stream or by organic solvents Removal was occasionally successful using a fine camel hair brush or by drawing the tip of the micropipette between the thumb and forefinger such relatively drastic cleaning techniques often resulted in damage to the tip A considerable time has been spent in an attempt to determine a cause for these caps as they were not observed in early experiments of the series or in a previous investigation of the lateral geniculate nucleus (Curtis and Davis 1962 1963) Caps have also been observed upon pipettes used for recent investigations upon spinal and cortical neurones Various types of hard glass have been tested different methods of cleaning were tried retaining potentials were not used in case the constant electrophoretic currents aided the formation of the caps chemical compounds from various sources were employed and animals of different ages were investigated None of these measures was successful and the cause of this disturbing occurrence remains unknown

The presence of these tissue components attached to the multibarrel micropipettes raised two important problems In the first place it has been observed in this present investigation that the excitatory actions of ACh and other cholinomimetics had a comparatively slow onset (see below) That this was not due to the obstruction of the access of these substances to the cells was proved by the finding of similar slow onsets when such caps were not present It may be argued that caps were always present and that pipettes not showing them were desheathed as they were withdrawn from the tissue However the time course of action of cholinomimetics could always be compared with that of acidic amino acids and many cells were investigated under circumstances where the

effects of the cap were either absent or minimal The other problem arises from the possibility that observed alterations in neurone behaviour might arise indirectly by drug action upon nearby structures either neuronal or even vascular It is conceivable that alterations in the firing rate of the neurone which gave rise to the largest extracellular spike potential but which was shielded from drugs ejected from pipette orifices by a cap could be produced synaptically by drug induced activity of neighbouring neurones, or even though less likely as a consequence of alterations in blood vessel calibre induced by ejected substances *When capping was present it was frequent to observe that the* firing of the major cell was preceded by that of other cells having smaller spike potentials. It is unlikely however that the observed drug effects were produced indirectly for the following reasons

(i) Observations were made on many neurones which responded to ejected drugs without the prior firing of other cells
(ii) In those cases where excitation was preceded by the firing of other neurones all of the cells were usually of a similar functional type and were classified as somatosensory relay cells (Andersen Eccles and Sears 1964) In some cases a major neurone for one position of the micropipette orifice became a minor unit when the tip was advanced slightly yet still responded to cholinomimetics
(iii) Thalamic neurones were fired both by choline esters and by excitant amino acids It is unlikely that these compounds affect blood vessels in a similar fashion.
(iv) Cholinoceptive neurones were found in close proximity to those unaffected by ACh thus rendering a vascular origin of the excitation unlikely

Results

Two types of thalamic neurone were investigated and records were obtained from 348 cells in 17 preparations The most common type of cell (75 %) was fired synaptically by impulses in only one afferent nerve The spike potentials were usually repetitive (Rose and Mountcastle 1954) and superimposed upon a negative field potential generated by nearby cells The minimal latency of firing was of the order of 6—8 msec after maximal stimulation of contralateral forelimb cutaneous nerves Occasionally cells of this type responded to volleys in several cutaneous nerves which had related peripheral fields but one nerve always evoked responses of shorter latency than did the others Many of these neurones were also fired antidromically by stimulation of the ipsilateral primary sensory cortex the spikes were of all or none character and had latencies of approximately 1 msec. These thalamic relay neurones were located within VPL and VPM and as has been reported previously (Mountcastle and Henneman 1949 Gaze and Gordon 1954 Poggio and Mountcastle 1960) neurones responding to stimulation of forelimb and facial nerves were usually located medially to those activated by hind limb nerves The other type of neurone *(25 %) tended to be slightly more superficial to the relay cells* and was fired by impulses in 2 or 3 afferent nerves Again firing was repetitive with a longer train of spikes than the other type and the latency usually exceeded 8 msec for cells responding to fore limb volleys Since these cells could not be fired antidromically by cortical stimulation it is unlikely that their axons pass to the primary sensory cortex and they have been presumed to be either intra-

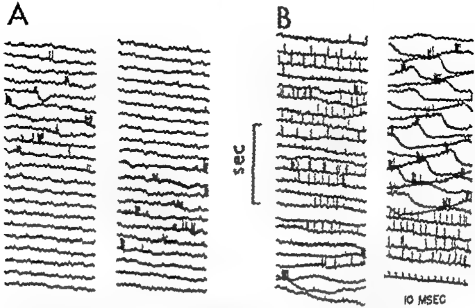

Fig 2 Potentials recorded extracellularly near a thalamic neurone (negativity upwards) and photographed upon film moving parallel to the Y axis of the oscilloscope There was approximately 2 msec between sweeps each sweep occupied approximately 135 msec
A — spontaneous firing recording amplifier time constant 30 msec
B — firing induced by the electrophoretic ejection of DL-homocysteic acid (22 nA) Recording amplifier time constant 3 sec
Time marker — 10 msec 1 sec for rate of movement of the film

thalamic interneurones (see also Andersen *et al* 1964) or neurones within the posterior group of nuclei (Poggio and Mountcastle 1960) having large receptive fields The percentages of these various cell types given above do not reflect the natural distribution of the cells since the prime aim of the investigation was to determine the chemical sensitivity of the thalamic relay neurones Accordingly many of the more superficial cells located by the electrode were not investigated fully

Occasionally difficulty was found in selecting a particular neurone out of the many which were fired synaptically Due to the close packing of neurones within the ventrobasal thalamus records were often obtained of two to four spike potentials of almost equal amplitude These cells could usually be separated by their different thresholds for synaptic excitation but separation was not so readily obtained when they were activated chemically either by an excitant amino acid or by a choline ester In order to analyse the behaviour of one of these neurones it was often necessary to move the electrode slightly so that one of the spike potentials was larger than the others It was thus possible to trigger selectively the pulse generator of the counting system with the larger spikes but care had to be taken to monitor spike shapes continuously upon the oscilloscope particularly when activating neurones with amino acids

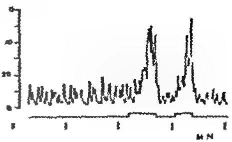

Fig 3 Frequency of firing of the cell illustrated in Fig 2 and plotted by means of a paper recorder Ordinate — spikes per sec Abscissa — time in min The marker beneath the frequency trace indicates two electrophoretic ejections of DL-homocysteic acid (22 nA) The spikes of Fig 2A were photographed during the early portion of this figure those of Fig 2B during the first ejection of DL-homocysteic acid

With many of these substances and with firing rates which exceeded 50—60 per sec there was an alteration in spike shape the negative positive extracellular spikes became less negative and more positive (cf. Curtis Phillis and Watkins 1960)

Spontaneous activity

Throughout these experiments spontaneous activity of thalamic neurones interfered with an accurate assessment of the temporal parameters of drug induced firing Contrary to earlier reports (Dempsey and Morison 1943 Morison Finley and Lothrop 1943) and in confirmation of more recent investigations (Galambos *et al* 1952 Andersen and Sears 1964) many neurones in the lateral thalamic projection nuclei displayed spontaneous spindles Each spindle consisted of a series of spikes or bursts each group containing 1—10 spikes of frequency 100—300 per sec and the groups had a frequency of 5—12 per sec (Fig 2A) These spindles of a particular cell (see Verzeano and Calma 1954) recurred so that when the discharge of the cell was displayed on the paper recorder the firing was periodic with a frequency of 8—16 per min Fig 3) An analysis of the spontaneous activity of several cells was carried out with a recording system which had a long time constant (1 sec) The spikes of each burst were followed by a prolonged positive wave which was often accentuated by the ejection of an excitant amino acid (Fig 2B) Under these circumstances the overall frequency of firing was increased but during the spindles the average frequency fell because during these positive potentials the firing was interrupted and groups of spikes occurred at a frequency of 5—10 per sec Fig 2B and 3 These observations suggest that this type of spontaneous activity in the thalamus is the result of an interaction between excitatory and inhibitory synaptic influences the observed positive potentials are of similar magnitude and time course to those which follow excitation of the cell by peripheral or cortical stimulation (see Andersen and Eccles 1962 and which have been shown to be due to large inhibitory postsynaptic potentials of the thalamic neurones Andersen Brooks and Eccles 1963 A more detailed study will be reported elsewhere (Andersen and Sears 1964 and it will be sufficient for present purposes to indicate the difficulties that such activity caused when attempt

were made to determine the onset and offset of drug induced firing and the frequency of discharge evoked by an excitant (see Fig 3 4 5) Pharmacological studies carried out on this spontaneous activity are reported in the following paper

In other neurones the spontaneous firing was of a more random nature but often, when firing was produced by the electrophoretic ejection of an excitant periodic fluctuations in frequency occurred not unlike those observed with cells exhibiting spontaneous spindles (see Fig 6A)

Excitation by amino acids

An intensive investigation of the amino acid sensitivity of thalamic neurones was not carried out As with spinal interneurones DL homocysteic acid proved to be a more powerful excitant than L glutamic acid (Curtis and Watkins 1963) and a pipette containing a 0 2 M solution of the sodium salt (pH 8 0—8 4) was used routinely in order to test the excitability of thalamic neurones N methyl D aspartic acid was even more powerful than either of these amino acids In contrast to the recently reported findings of Krnjevic and Phillis (1963a) upon cortical neurones L (+) glutamic acid was only once and a half to twice as potent as D (—) glutamic acid an observation which is similar to that made on spinal cells (Curtis and Watkins 1963)

Excitation by acetylcholine

Practically all of the thalamic neurones which were tested with ACh were und to be excited by this substance This was in marked contrast to the in itivity of cells located in areas more superficial and deeper to VPL and VPM which were readily fired by acidic amino acids but did not respond to cutaneous volleys In very many cases particularly with thalamic relay neurones respond ing synaptically to volleys in only one cutaneous nerve the depolarization which re ulted from ACh administered with current of 5—80 nA was sufficient to produce spike potentials and the firing frequency could be measured directly The sensitivity of these cells to ACh appeared to be similar to that of spinal Renshaw cells (Curtis and Eccles 1958a) In other cases the depolarization re mained subthreshold in spite of the use of electrophoretic currents of 100—150 nA but was readily revealed when the excitability of the neurones was tested with either an excitant amino acid or a just threshold synaptic volley These latter neurones were usually thalamic interneurones which were excited synaptically by volleys in two or more cutaneous nerves to give a long train of discharges and which were not antidromically fired by cortical stimulation ACh sensitive cells of both types were located in all preparations irrespective of varying degrees of anesthesia In several cases the i v administration of small doses of pentobarbital (3—10 mg/kg) converted random spontaneous firing to spindles and reduced the sensitivity of thalamic neurones to both ACh and DL homocysteic acid

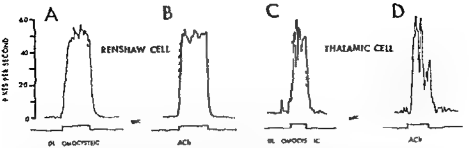

Fig 4 Frequencies of firing of a Renshaw cell (A, B) and a thalamic neurone (C D) induced by DL-homocysteic acid (A ?8 nA, C-4 nA) and acetylcholine (B-35 nA D 30 nA) Abscissae—time in [illegible] sec periods The electrophoretic ejections are signalled by markers beneath the time indicators The broken lines in each record show the response of the frequency recording [illegible] a 50 spike per sec calibrating signal

The characteristics of the excitation of thalamic neurones by ACh differ from those of spinal Renshaw cells although there were no essential differences between the responses of these cells to excitant amino acids These points are illustrated in Fig 4 in which are plotted the frequencies of firing of a Renshaw cell (A B) and a thalamic relay neurone (C D) evoked by DL homocysteic acid and acetylcholine (ACh) The responses of the thalamic neurone were also filmed and sections of these records are reproduced in Fig 5 the symbols in Fig 5 A and C indicating the onset of electrophoretic current flow those in B D its termination The onset of firing of both neurones by DL-homocysteic acid was comparatively slow and in the case of the thalamic neurone although the onset of amino acid firing is difficult to distinguish from the spontaneous discharges there is a latency of approximately 2 1/2 sec Similarly the rate of onset of firing of the Renshaw cell was slow and the peak frequency of about 50 spikes per sec did not occur until 8 sec after the onset of current flow In both cases these ejections were made from micropipettes upon which minimal retaining voltages had been maintained and out of which several ejections had been made immediately preceding those which are illustrated These circumstances ensured that the onset of drug induced firing was minimal (see Curtis and Watkins 1963) and thus the delay was unlikely to be associated with the extrusion of the amino acid from the micropipette At the termination of the current flow the cessation of firing of both neurones was delayed and in the case of the thalamic neurone firing continued for approximately 4 3 sec These slow rates of onset and offset of firing induced by DL-homocysteic acid are similar to those observed with spinal interneurones (Curtis and Watkins 1963) and are in marked contrast to the rapid onset and cessation of firing produced by L-glutamic acid (Curtis *et al* 1960 Curtis and Andersen 1962) As with spinal interneurones and Renshaw cells the firing of thalamic neurones by L-glutamic acid usually began within 200 msec of the onset of current flow

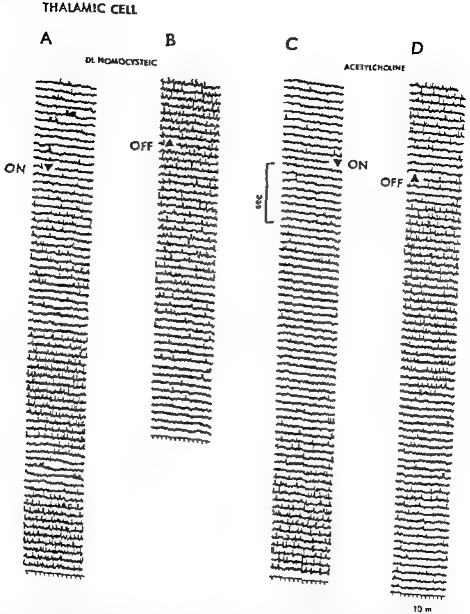

Fig 5 Filmed records as in Fig 2 of the spike potentials of the thalamic neurone of Fig 4 C and D Each column is to be read from above downwards The black triangles signal the onset and offset of electrophoretic ejections A, B — DL-homocysteic acid (Fig 4C) C D acetylcholine (Fig 4D)

Timer — 10 msec for all records 1 sec for rate of movement of the film

and ceased within a similar time interval of its termination (Fig 6A) There was thus little or no difference between the time characteristics of the excitation of spinal and thalamic neurones by these different amino acids

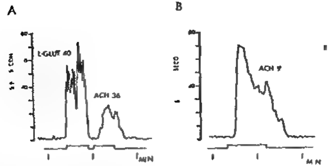

Fig 6 The frequencies of firing of two thalamic neurones are plotted with respect to time (minutes)
A — firing induced by L-glutamic acid (L-Glut — 40 nA) and acetylcholine (ACh — 36 nA)
B — firing induced by acetylcholine 9 nA
In each case electrophoretic ejections are signalled by the marker beneath the frequency trace

In contrast, there was a considerable difference between the excitant action of ACh upon the two types of neurone When ACh was administered to the Renshaw cell, firing commenced within 200—300 msec and the maximal firing frequency was attained within 1—2 sec (Fig 4B) At the termination of the electrophoretic current firing ceased within 500 msec The onset of firing of thalamic neurones by ACh was invariably delayed and in the case of the cell illustrated in Fig 4D a period of 4 1/2 sec elapsed before firing commenced (see Fig 5C) With other cells this latent period ranged between 2 and 30 sec (Fig 6A B) In many cases when the spontaneous spindle bursts were present this spontaneous firing was temporarily depressed during this interval (see Andersen and Curtis 1964) The actual frequency of firing of thalamic neurones by ACh was usually difficult to assess and in some cells the discharge rate often fell during the ejection of the excitant by a constant electrophoretic current This is apparent in the records of Fig 4D and 6A but is more obvious in Fig 6B This latter cell had a very low spontaneous rate of firing which increased 9 sec after the onset of the electrophoretic current to a firing rate of 70 spikes per sec Thereafter the firing rate fell gradually in spite of a constant current flow of 9 nA through the ACh containing barrel This fall in frequency was not associated with any change in the spike height or shape and since it was not observed to such a degree with other nearby cells using the same micropipette is unlikely to have been associated with a diminution in the rate of ACh ejection throughout the period of current flow In some instances the sensitivity of thalamic neurones fell with repeated ejections On several occasions cells which were readily fired by the first administration of ACh using currents of the order of 20—50 nA for periods of 30—60 sec rapidly became insensitive so that thereafter the excitant action could be detected only by the

simultaneous administration of an amino acid the sensitivity to which remained unaltered throughout the testing period Most cells however did not display such changes in sensitivity and it was possible to obtain reasonably constant maximal firing rates by repeated periods of ejection This permitted an analysis to be made of the action of blocking and potentiating drugs upon the ACh evoked responses of thalamic neurones (see Andersen and Curtis 1964)

When the electrophoretic current which ejected ACh near a thalamic neurone was terminated there was invariably a delay in the cessation of firing Because of the spontaneous activity the actual time over which firing ceased was often difficult to measure but periods of 5—60 sec were observed In the case of the cell illustrated in Figs 4 and 5 the time was approximately 5 1/2 sec whereas the cells of Fig 6 continued to fire for approximately 14 sec (A) and 30 sec (B) after the electrophoretic currents ceased to flow

These prolonged latencies of firing and cessation of firing were unlikely to have been due to differences between the time courses of the ejection of ACh from micropipettes in the vicinity of thalamic neurones and Renshaw cells Although the latencies of onset and the attainment of maximal firing frequency of thalamic neurones could be shortened slightly by repeated ejections at intervals of 10—30 sec and by a reduction in the retaining potential which presumably reduced the delay between the onset of current flow and the ejection of ACh (Curtis and Watkins 1963) the pattern of firing obtained with Renshaw cells (Fig 4A) could never be reproduced The minimal latency of firing invariably exceeded 2 sec Furthermore the use of an excessive retaining potential never reduced the period over which firing ceased to the short intervals which were observed with Renshaw cells Thus after diffusion due to the uncontrolled efflux of ACh (Castillo and Katz 1957) is unlikely to be an explanation of the prolonged firing of thalamic neurones In general there was a rough correlation between the minimal latency of firing and the time of offset In most cases where firing persisted for more than 10 sec after the ACh ejection ceased the latency of firing was similarly prolonged When the latency was of the order of 2—5 sec the offset time rarely exceeded 10 sec but in other cases when firing ceased within 5—10 sec the onset of firing was comparatively slow In many of these instances the firing was preceded by a reduction in the spontaneous discharge rate (see Fig 10 Andersen and Curtis 1964) in others the onset of firing evoked by DL homocysteic acid was also delayed and it is thus probable that tissue caps upon the micropipettes hindered the diffusion of ejected substances to the neurones

Discussion

Although it may be assumed that the depolarization of thalamic neurones by ACh is the result of an interaction between this substance and receptors which are located upon pre or postsynaptic structures a discussion of the

nature of these sites must await the pharmacological analysis presented in the following paper However the difference in the sensitivity of the two functional types of thalamic neurone which presumably results from the number and accessibility of suitable receptor sites suggests that the ACh receptors are related to synaptic processes

It is pertinent to enquire into possible explanations for the slow onset of the firing and the prolonged duration which follows the termination of the ACh ejection These times stand in marked contrast to the short onset and offset when Renshaw cells were fired by electrophoretically administered ACh (Curtis and Eccles 1958a Curtis Phillis and Watkins 1961) Since electrophoretic currents of similar magnitude were used to eject ACh from solutions of the same concentration into the vicinity of these two types of neurone it is unlikely that differences in the relationship between the current pulse and the ejection of the drug account for the observed time differences The major portion of the latency of firing would include the time taken for the ejected ACh to reach the appropriate receptor sites in adequate concentration together with the time necessary for the interaction between ACh and the receptors to become sufficiently effective to produce a membrane depolarization which exceeded the threshold for spike initiation In the case of thalamic neurones an additional complication was introduced by the occasional observation of a preliminary depression of the spontaneous discharges by ACh This depression which has never been observed prior to the excitation of Renshaw cells presumably delayed the onset of firing by increasing the apparent threshold for excitation Factors to be considered in regard to the cessation of the firing when the ejecting current is terminated include the rate of diffusional reduction in the ACh concentration the rate of enzymic and of other types of inactivation and the rate of dissociation of the ACh receptor complex

It is possible that partial obstruction of the orifices of micropipettes by tissue components accounts in part for the slow action of ACh upon thalamic neurones but in many cases there was no slowing of the onset of firing by an excitant amino acid ejected from adjacent barrels of the same micropipette Furthermore micropipettes used in recent experiments upon Renshaw cells have also been observed to be capped by cellular debris (D R Curtis and R W Ryall — unpublished observations) yet there has been little or no delay in the onset of firing of these cells by ACh These observations suggest that the presence of such caps was not wholly responsible for the difference between the responses of thalamic and Renshaw cells

It is generally assumed that extracellular spike potentials are recorded in the vicinity of the soma initial segment and portion of the bases of the larger dendrites of nerve cells If the structure of thalamic neurones was such that the ACh sensitive receptors were located upon dendrites at some distance from the spike generating areas of the cell the time taken for ACh to diffuse to these receptors from its site of ejection close to the recording microelectrode could

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From the Department of Physiology Australian National University Canberra Australia

The Pharmacology of the Synaptic and Acetylcholine-Induced Excitation of Ventrobasal Thalamic Neurones

By

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Abstract

P Andersen and D R Curtis *The pharmacology of the synaptic and acetyl choline induced excitation of ventrobasal thalamic neurones* Acta physiol scand 1964 61 100—120 — The pharmacological properties of acetylcholine (ACh) *sensitive neurones in the ventrobasal nucleus of the thalamus of cats anes* thetised with pentobarbital sodium have been investigated by the electro phoretic method of ejecting substances from multibarrelled micropipettes *Carbamoylcholine acetyl β methyl choline* dl muscarine and nicotine were effective excitants when compared with ACh other choline esters and related compounds were less active Physostigmine and neostigmine increased the effectiveness of ACh and in addition were excitants The excitatory action of ACh was effectively abolished by dihydro-β-erythroidine and by atropine the action of atropine however was not very specific It is proposed that the sensitivity of these neurones to cholinomimetics indicates that ACh normally functions as an excitatory transmitter within the thalamus the synaptic pathway involved has not yet been identified Cholinomimetics 5 hydroxy tryptamine and some related substances also depress the excitability of thalamic neurones

In the preceding paper it has been reported that neurones in the ventrobasal complex of the feline thalamus which are fired synaptically by volleys in cutaneous sensory nerves are also excited by electrophoretically administered ACh (Andersen and Curtis 1964) The greater sensitivity of thalamic relay neurones compared with that of thalamic interneurones suggested that this sensitivity to ACh may be related to synaptic mechanisms Although complete figures are not available for the various thalamic nuclei of the cat the moderate amounts of ACh choline acetylase and cholinesterases in the

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Table I Compounds found to be excitants of thalamic neurones

Group	Compound	Potency[1]
(A) *Simple quaternary ammoniums and esters of choline*	Tetramethylammonium bromide	+(+)
	Tetraethylammonium bromide	+
	Acetylcholine bromide	++
	Propionylcholine iodide	+
	Butyrylcholine iodide	+
	Acetyl β methylcholine chloride	+(+)
	Acetylhomocholine chloride	+
	β Carbomethoxyethyl trimethylammonium chloride	++
	Carbamoylcholine chloride	+++
	Succinylcholine bromide	+
(B) Complex quaternary *ammoniums*	Neostigmine bromide	+(+)
	1 6-Hexane bis (*trimethylammonium*) iodide	(+)
(C) Muscarine and derivatives	dl Muscarine chloride	+(+)
	D(−)Muscarone chloride	(+)
(D) Miscellaneous	Arecoline hydrobromide	+
	Gallamine triethiodide	+
	Nicotine hydrochloride	+(+)
	Pilocarpine hydrochloride	(+)
	Physostigmine sulphate	+

[1] Potency expressed relative to that of ACh ++ (see text)

5196 — 28 — X — 65

mammalian thalamus (MacIntosh 1941 Feldberg and Vogt 1948 Burgen and Chipman 1951 1952 Koelle 1954 Zettler and Schlosser 1955 Hebb and Silver 1956 Hebb 1957 Gerebtzoff 1959) support the proposal that ACh has a transmitter function within the cutaneous relay nuclei (Feldberg and Vogt 1948) However confirmation of this function of ACh requires the demonstration that the pharmacology of synaptic excitation be identical with that of the excitation by ACh Consequently experiments were designed to determine the nature and possible location of the cholinoceptive sites upon thalamic neurones and to investigate the pharmacology of synaptic excitatory processes within the ventrobasal complex The methods have been described in detail in the preceding paper

Results

Excitatory action of cholinomimetics and related compounds

In the present investigation various cholinomimetics were compared with ACh as excitants of thalamic neurones When practicable these agents were passed electrophoretically from solutions of equal molarity (generally 0 1 M) and it was assumed that the rates of ejection of different compounds were then

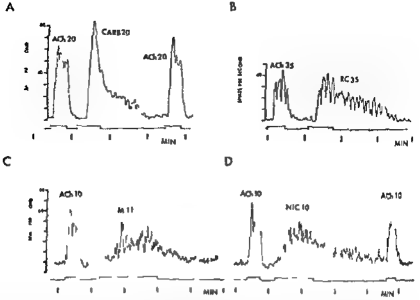

Fig 1 In this and in most other figures of this paper the frequency of firing of thalamic neurones was plotted by means of a rate meter and paper recorder Drug ejections are signalled either by means of marker pens at the top and/or bottom of the traces or by solid horizontal lines Time in minutes is indicated beneath the frequency traces
A — firing of a thalamic neurone evoked by two ejections of acetylcholine (ACh – 20 nA) and one of carbamoylcholine (Carb 20 nA)
B — firing of another thalamic neurone in response to acetylcholine (ACh — 35 nA) and β carbomethoxy ethyl trimethylammonium (RC — 35 nA)
C D — firing of a thalamic neurone evoked by acetylcholine (ACh — 10 nA) dl muscarine (M — 11 nA) and nicotine (NIC — 10 nA)

proportional to current flow It must be realised however that equal rates of ejection do not necessarily produce equal extracellular concentrations even when several compounds are ejected at the same distance from a particular neurone Thus differences in the potencies of two substances may be associated with differences in extracellular concentrations as a result of enzymic inactivation or adsorption upon tissue components rather than to differences in effectiveness at neuronal receptor sites

The compounds of Table I are arranged according to their structures and the potencies as excitants of ventrobasal thalamic neurones are expressed relative to that of ACh The number of symbols is not a direct measure of potency but indicates greater +++ or less + activity than ACh ++ This activity was measured either by the frequency of firing when the depolarization was adequate to fire neurones or by the amount of potentiation when depolarization was detected by the use of amino acid induced firing (see Curtis and Davis 1963) Substances more active than ACh invariably produced greater rates of firing when ejected with the same electrophoretic current as that used

to administer the reference compound Comparisons were usually made upon 5—10 cells in at least three different preparations and new multibarrelled micropipettes and fresh solutions were used for each animal The enclosure of a symbol in brackets indicates that there is some doubt concerning its true value, but in these cases the order of the activity was such that further investigation was not warranted In general the maximum cationic electrophoretic currents were of the order of 100 nA and substances which when ejected with this current for 60—90 sec neither fired neurones nor facilitated the firing evoked by the simultaneous ejection of an excitant amino acid were rated zero 0

As shown in Table I, many choline esters and other cholinomimetics excited thalamic neurones In general the latency from the commencement of the electrophoretic current to the attainment of peak firing frequency and the duration of excitation after termination of the current flow exceeded the respective values for ACh Carbamoylcholine was a more potent and prolonged excitant than ACh, and results from one cell are shown in Fig 1A With other cells there was a greater difference in potency between these compounds (of the order of a factor of 2) but in general the potency differences was not as great as has been observed with Renshaw cells (Curtis and Ryall 1964) β Carbomethoxy-ethyl trimethylammonium (reversed carboxyl analogue of ACh — Bass Schueler Featherstone and Gross 1950) was as active as ACh (Fig 1B) whereas dl muscarine was slightly less potent (Fig 1C) When tested upon spinal Renshaw cells the first of these compounds was much more effective than ACh but dl muscarine was a very weak excitant (Curtis and Ryall 1964) *On the other hand D( )muscarone a more powerful excitant of Renshaw cells* that dl muscarine (Curtis and Ryall 1964) was an extremely weak excitant of thalamic neurones and in addition depressed the sensitivity to ACh Both tetramethylammonium and tetraethylammonium excited thalmic neurones and this again contrasts with the action of these substances upon Renshaw cells where tetramethylammonium was a more potent excitant than ACh and tetraethylammonium reduced the sensitivity to ACh (Curtis and Ryall 1964) Other choline esters including propionylcholine *n* butyrylcholine acetyl homocholine and succinylcholine were less active than ACh On the other hand acetyl β methylcholine was almost as potent as ACh a finding which contrasts with the low potency of this ester as an excitant of Renshaw cells (Curtis and Ryall 1964)

The action of nicotine was of considerable interest in view of the high activity of this alkaloid as an excitant of Renshaw cells (Curtis and Eccles 1958a) In contrast this substance was less potent than ACh as an excitant of thalamic neurones (Fig 1D) although as with Renshaw cells its excitant action was more prolonged than that of ACh Both arecoline and pilocarpine were less potent than ACh and in addition pilocarpine partially blocked the action of subsequent doses of ACh

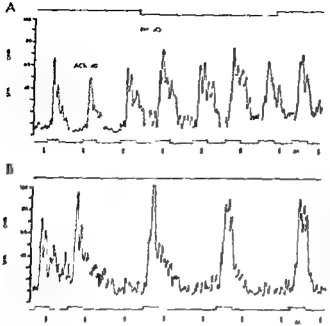

Fig 2 A — Frequency of firing of a thalamic neurone in response to periodic ejections of acetylcholine (ACh — 40 nA) as indicated by the lower signal trace During the time indicated by the upper signal trace physostigmine was ejected from another barrel of the five barrelled micropipette (PHYSO — 10 nA) B — continuous with A.

Action of cholinesterase inhibitors

It was hoped that the use of inhibitors of cholinesterase would permit the detection of enzymes associated with the inactivation of transmitters released upon thalamic neurones although one explanation of the relatively prolonged excitatory action of ACh would be that cholinesterases in this area were not very effective in hydrolysing this ester

Physostigmine facilitated and prolonged the excitatory action of ACh and in addition was a weak excitant Fig 2 plots the frequencies of firing of a thalamic neurone evoked by ejections of ACh (40 nA for 20 sec) as indicated by the lower signal trace Physostigmine was ejected from another barrel of the micropipette as a cation (10 nA for 210 sec upper signal trace) and subsequently ACh became a more effective excitant with a more prolonged action The tracing of Fig 2B is continuous with that of Fig 2A and shows clearly the prolongation of the ACh excitation and its gradual recovery Unfortunately *this cell was damaged by the micropipette before full recovery took place* The rise in firing frequency between the last six ACh ejections of Fig 2A and the first two of Fig 2B may have been due to an excitant action of physostigmine but may equally have been produced by too frequent administrations of ACh Fig 3 plots results from a neurone in another preparation Alternate 23 sec ejections of ACh (10 nA) and DL-homocysteic acid (10 nA) were made from different barrels of a micropipette there was a period of 30 sec between ejections The symbols indicate the maximum firing frequency produced by each sub-

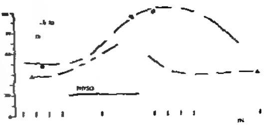

Fig 3 Each point plots the maximal frequency of firing of a thalamic neurone in response to ejections of acetylcholine (ACh 10 nA for 23 seconds ●) and DL-homocysteic acid (DLH 10 nA for 23 seconds ▲) Physostigmine was ejected from another barrel of the five barrel electrode (PHYSO — 7 nA) for 5 3 minutes (lower bar)

stance and the lower bar indicates the time over which a cationic current of 7 nA ejected physostigmine from a third barrel The frequency of firing evoked by ACh was doubled and in addition but not shown in this figure the duration of action of ACh was increased Furthermore in addition to this effect which presumably was produced by partial inhibition of cholinesterases in the vicinity of the neurone physostigmine also increased the firing rate produced by DL homocysteic acid This latter result suggests a direct excitant action of physostigmine which had a briefer duration than the anticholinesterase effect Similar increases in the frequency evoked by the amino acid were observed in other cells in the absence of ejections of ACh With several cells physostigmine was ejected with currents exceeding 30 nA and was observed actually to depress the action of ACh whilst enhancing the excitatory action of DL homocysteic acid or L glutamic acid It was usually impossible to pass currents greater than 30—50 nA through micropipettes which contained 0 1M physostigmine sulphate and which had electrical resistances of 40—200 MΩ

Neostigmine was a relatively potent excitant of thalamic neurones (Table I) and it was thus difficult to determine whether the associated potentiation and prolongation of the firing produced by ACh was due to this direct effect or to the inhibition of cholinesterase

In view of the excitatory effects of these anticholinesterase agents it was difficult to ascertain whether they prolonged any form of synaptic excitation of thalamic neurones by inhibiting cholinesterase With both physostigmine and neostigmine there was little or no increase in the number of spikes within spontaneous spindles or in the firing evoked by cutaneous volleys or in the burst responses which followed cortical or peripheral stimulation This evidence suggests that it is unlikely that any of these types of synaptic excitation is cholinergic since the slight potentiation which was seen could have been associated merely with the direct excitation produced by these agents It is usually possible when ejecting neostigmine (or edrophonium) near spinal Renshaw cells to select a small electrophoretic current which produces a concentration of the anticholinesterase sufficient to enhance synaptic and ACh excitation and yet not increase the background firing rate of these neurones With larger currents this background rate of discharge can be increased

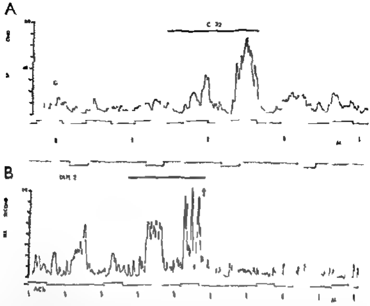

Fig 4 A — firing frequency of a thalamic neurone produced by ejections of L glutamate (L GLUT 38 nA lower signal trace) (+) Tubocurarine was ejected (DTC — 2? n \) over the time indicated by the horizontal line
B — Frequency of firing of another thalamic neurone in response to acetylcholine (ACh 70 n\ lower signal) and DL homocysteic acid (DLH 20 nA upper signal and ●) Gallamine was ej cted during the time indicated by the horizontal line (10 nA)
Note different time scales for A and B

) Although physostigmine readily penetrates the blood brain barrier (Eccles Fatt and Koketsu 1954) it was not considered practicable to administer it systemically because a reliable assessment of its mode of action would be prevented by the associated side effects upon muscles the cardiovascular and respiratory systems together with the possibility that there may be changes in transmission at other synap es upon polysynaptic pathways converging upon thalamic neurones

Action of acetylcholine antagonists

A series of compounds known to block the action of ACh at peripheral cholinoceptive sites were tested upon the spontaneous, synaptic and acetyl choline evoked spikes of thalamic neurones Many of these compounds proved to be excitants, and were thus unsuitable for the detection of the nature of synaptic transmitters

(+) Tubocurarine ejected with currents of 10—100 nA readily excited thalamic neurones and facilitated the firing evoked by an excitant amino (Fig 4A) In several cells evidence was obtained that this substance depres. the excitatory action of ACh but such depression was not cle- methonium also proved to be an excitant and in several cells sh

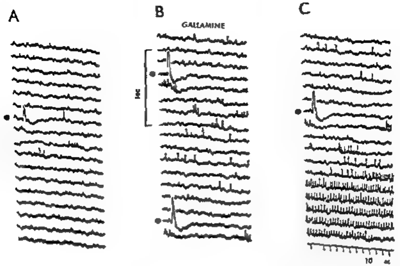

Fig. 5 Spike responses of a thalamic neurone photographed on film moving at right angles to the oscilloscope beam. The symbol ● indicates beams during which the contralateral median nerve was stimulated.
A — control responses
B — 20 seconds after a current of 50 nA began to eject gallamine; the ejection continued throughout B and C
C — continuous with B
Voltage calibration — individual spikes approximately 350 μV in amplitude
Time scale — 10 msec for oscilloscope beam; sec for movement of film.

the action of ACh whilst simultaneously the action of excitatory amino acids was enhanced. Gallamine triethiodide was administered to 18 cells in seven different preparations and in all cases had a characteristic excitant action. After an initial delay of 10—90 sec, which depended upon the magnitude of the electrophoretic current, cells were depolarized. This was revealed either as actual firing or as an enhancement of amino acid and ACh induced firing, as illustrated in Fig. 4B. Eventually gallamine alone, or with the addition with another excitatory agent, produced characteristic bursts of high frequency firing in which there was a reduction in spike height which progressed to complete block. Thereafter the cell was totally unresponsive to any excitatory agent for several minutes, due no doubt to a prolonged depolarization which inactivated the spike generating mechanism. When small currents were used to eject gallamine, as in Fig. 4B, the high frequency bursts of firing, which occurred at the time indicated by an arrow, were occasionally followed by an apparent block of the excitatory action of ACh, whereas DL homocysteic acid still produced firing. This was seen but rarely, and may be associated merely with the different potencies of these two substances as excitants, rather than with a specific anticholinergic effect. Fig. 5 illustrates spike responses of a neurone which was excited by gallamine (50 nA). Control spontaneous spikes

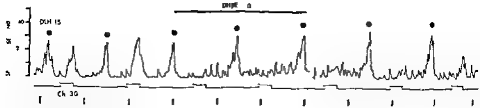

Fig 6 Frequency of firing of a thalamic neurone evoked by DL-homocysteic acid (DLH 15 nA ●) and acetylcholine (ACh 30 nA lower signal) Dihydro-β erythroidine (DHβE) was ejected with a current of 20 nA

are shown in Fig 5A together with spikes evoked synaptically by stimulation of the median nerve (●) These latter spikes are not readily apparent because of their superposition upon a large negative field potential Within 20 sec of ejecting gallamine additional spikes were evoked (Fig 5B) and the field potential was enhanced Three seconds later a prolonged burst of firing was observed (Fig 5C) in which spike height was reduced and eventually the spike was blocked Thereafter the field potential was also reduced in magnitude for approximately 20 sec after the gallamine ejection was terminated This type of response which is presumably due to a direct action of gallamine upon neuronal membrane is very similar to that observed in the medullary reticular formation (Salmoiraghi and Steiner 1963) and recent experiments have indicated that gallamine also excites non cholinoceptive spinal interneurones and cholinoceptive Renshaw cells (Curtis and Ryall 1964) and cortical neurones (Crawford and Curtis 1964) in a similar fashion An earlier finding (Curtis Phillis and Watkins 1961) that gallamine reduced the effectiveness with which electrophoretically administered ACh or ventral root volleys excited Renshaw cells requires correction since in these experiments the firing frequency of Renshaw cells was not observed continuously and thus the high frequency excitation which preceded a depolarization block was overlooked

Dihydro β erythroidine (DHβE) is a cholinergic blocking agent of considerable interest because it penetrates the blood brain barrier and after systemic or local administration depresses the responses of spinal Renshaw cells to both ACh and ventral root volleys (Eccles Fatt and Koketsu 1954 Eccles Eccles and Fatt 1956 Curtis and Eccles 1958b Brooks and Wilson 1959 Curtis et al 1961) without affecting responses evoked by excitant amino acids Dihydro β erythroidine readily depressed the excitation by ACh of the 27 thalamic cells (6 prep) to which it was administered The specificity of this block was demonstrated by the failure of DHβE to diminish firing produced by either L glutamic acid or DL homocysteic acid in many cases such amino acid induced firing was enhanced by DHβE Furthermore DHβE actually excited thalamic neurones particularly when ejected with currents of the order of 20—100 nA

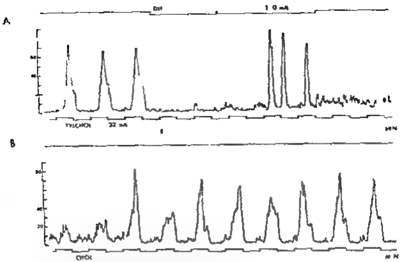

Fig 7 A — Frequency of firing of a thalamic neurone in response to periodic ejections of acetylcholine (lower signal 32 nA) Dihydro-β-erythroidine (DHβE) was ejected with a current of 75 nA which was increased to 100 nA (upper signal)
B — continuous with A

Fig 6 illustrates the effect of DHβE upon the firing of a thalamic neurone which was produced by ACh (30 nA lower signal trace) and DL homocysteic acid (15 nA ●) During the time indicated by the horizontal bar a cationic current of 20 nA ejected DHβE from another barrel of the micropipette which contained an 0 1M solution of DHβE hydrobromide The excitant action of ACh was suppressed and partial recovery is apparent towards the end of the figure Complete recovery was observed 3 min later In contrast the frequency of firing produced by DL homocysteic acid remained practically unaltered and in addition there was an increase in the background firing rate of the cell which subsided 5—6 min after the cessation of the DHβE ejection This excitation by DHβE is also illustrated in Fig 7 in which is plotted the frequency of firing of another thalamic neurone in response to ACh (32 nA lower signal trace) the tracing of Fig 7B is continuous with that of Fig 7A After 3 control responses a current of 75 nA ejected DHβE for 2 2 min thereafter the current was increased to 100 nA for a further 3 3 min (upper signal trace) Initially the ACh responses were completely blocked and then there was a slow increase in the rate of discharge of the cell with some bursts of firing which coincided with the last two ACh ejections before the DHβE current was terminated Thereafter this background firing subsided and the effectiveness of ACh gradually increased until almost complete recovery was observed 13—14 min later When similar currents have been used to administer DHβE near Renshaw cells the recovery of ACh firing was usually extremely prolonged and often exceeded 45 min (Curtis and Ryall 1964)

The ability of DHβE to block the excitatory action of ACh suggested that this substance would be suitable to test whether any synaptic excitation of thalamic neurones was cholinergic particularly *if low electrophoretic doses* were used to limit the amount of drug induced excitation The excitation of 13 neurones by cutaneous volleys was unaltered by concentrations of DHβE adequate to completely suppress firing induced by electrophoretically administered ACh Furthermore the spontaneous spindle activity of these neurones was unaltered and in several cases where repetitive bursts were elicited by cortical or peripheral stimulation (Andersen and Eccles 1962) these were also unchanged It is conceivable that many of the synapses involved in these various types of synaptic excitation were outside the volume of tissue affected by the electrophoretically administered DHβE although it is obvious that if cholinergic synapses were present some of them must have been with n range of ACh ejected from adjacent barrels of the same micropipette In order to overcome this difficulty comparatively large amounts of DHβE were administered i v (1 5 1 3 and 2 mg/kg in 3 different preparations) Such doses had no effect upon spontaneous spindles the spikes evoked from the periphery or from the cortex and field potentials generated by cutaneous stimulation

Considerable difficulty was experienced in demonstrating a specific action of atropine upon thalamic neurones It has already been demonstrated that atropine diminishes the excitant action of ACh upon spinal Renshaw cells and in addition in slightly higher concentrations reduces the excitation produced by an amino acid (Curtis and Phillis 1960) This latter non specific effect is presumably due to a local anesthetic type of action upon the spike mechanism of these cells and can also be demonstrated with other cholinoceptive and non cholinoceptive neurones (Curtis and Phillis 1960 Krnjević and Phillis 1963 a c) Although extremely small electrophoretic currents were used it proved impossible to selectively reduce the ACh sensitivity of thalamic cells without changing the sensitivity to an excitant amino acid In some cases the uncontrolled diffusion of atropine from pipettes of orifice 1 μ which contained an 0 01 M solution of atropine sulphate was sufficient to depress the excitation of cells by DL-homocysteic acid However in practically every case the depression of the ACh responses was more profound and of slightly longer duration than that of the amino acid responses The firing frequency of a thalamic neurone is plotted in Fig 8A and the responses were induced by alternate ejections of ACh (40 nA lower signal trace) and DL homocysteic acid (15 nA upper signal trace ●) After several control ejections of each of these two agents atropine was ejected by a cationic current of 5 nA from a barrel which contained an 0 1 M solution of atropine sulphate The firing produced by subsequent doses of ACh was reduced and eventually abolished whereas that produced by DL homocysteic acid was merely reduced The maximal rate of firing by DL-homocysteic acid reached a plateau 5 min after

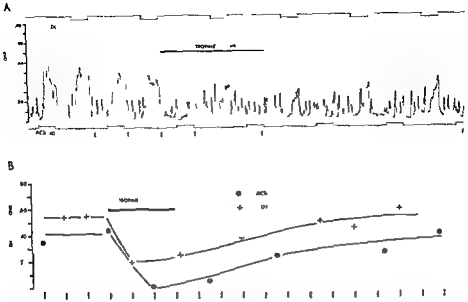

Fig 8 A — Frequency of firing of a thalamic neurone evoked by acetylcholine (ACh 40 nA lower signal) and DL-homocysteic acid (DLH 15 nA upper signal and ●) The ejection of atropine (5 nA) is signalled by the horizontal line
B — The symbols plot the maximum firing frequency of a thalamic neurone produced by alternate ejections of acetylcholine (ACh, 60 nA ●) and DL-homocysteic acid (DLH 4 nA, +) Atropine was ejected with a current of 5 nA for 3 minutes (horizontal line)

the atropine ejection ceased (final dose illustrated) whereas at this time the excitant action of ACh was still reduced Unfortunately full recovery of the ACh action was not observed due to accidental dislodgement of the micropipette 3 min later One feature which is clear in this figure is the failure of atropine to reduce the spindle activity of this neurone a finding which was confirmed in several other cells

Results from another cell in another preparation are plotted in Fig 8B In this case the thalamic neurone was excited by alternate ejections of ACh (60 nA) and DL-homocysteic acid (4 nA) each of 38 sec duration and the symbols plot the respective maximum firing frequencies which were so attained Atropine was administered with a current of 5 nA for 3 min and almost abolished the firing evoked by ACh but merely diminished that produced by the amino acid The sensitivity of this neurone to DL-homocysteic acid had recovered 6 1/2 min after the atropine ejection ceased and 5 min later there was almost complete recovery of the sensitivity to ACh

In general for the 18 cells (7 prep) upon which the interaction of atropine ACh and an excitant amino acid was tested as in Fig 8 there was a factor of only 2 to 4 between the recovery times of the depression of ACh firing and of the firing evoked by either DL-homocysteic or L glutamic acid In all cases despite

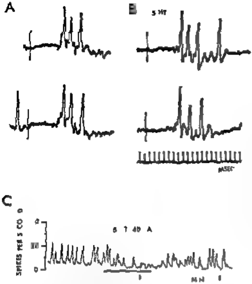

Fig 9 A B — Spikes of a thalamic neurone evoked by maximal stimulation of the contralateral ulnar nerve A — before B — 30 seconds after a current of 60 nA began to eject 5 hydroxytryptamine (5 HT) The spike preceding the stimulus artifact in the lower record of A was spontaneous Time — msec
C — Spontaneous firing frequency (spindles) of a thalamic neurone before during and after a current of 40 nA ejected 5 hydroxytryptamine (5 HT) from one of the barrel of a 5 barrel micropipette The 5 HT ejection is signalled by the horizontal line

complete abolition of ACh firing there was no effect upon spikes elicited synaptically by volleys in cutaneous nerves or upon spontaneous spindle activity Furthermore in several preparations atropine sulphate was administered i v (0 5—1 mg/kg) again without effect on synaptic responses

Other pharmacologically active substances

It was of particular interest to determine the action of 5 hydroxytryptamine (5 HT) and related substances upon neurones of the ventrobasal complex of the thalamus because these compounds readily depress the synaptic excitation of neurones in the lateral geniculate nucleus by optic tract impulses (Bishop et al 1960 Curtis and Davis 1962) Furthermore concentrations of 5 HT 4 HT 3 hydroxytyramine and ergometrine which were adequate to suppress the synaptic excitation of geniculate neurones were without effect upon the excitation of these cells by amino acids or ACh (Curtis and Davis 1963) In general the electrophoretic currents necessary to administer sufficient 5 HT to suppress the synaptic firing of geniculate neurones rarely exceeded 50 nA and in most cases a current of 5—10 nA was adequate These results are in marked contrast to those observed upon cells of the cerebral cortex since Krnjevic and Phillis (1963d) have reported that 5 HT 4 HT and 3 hydroxytyramine depress the synaptic spontaneous and amino acid excitation of these neurones

5 HT was administered to 32 neurones in the ventrobasal thalamus (7 prep) and in no case could the short latency synaptic excitation of these cells by cutaneous volleys be depressed (Fig 9A B) The maximum electrophoretic currents used were of the order of 50—70 nA a limitation which resulted from the comparatively high resistance of micropipettes which contained a saturated

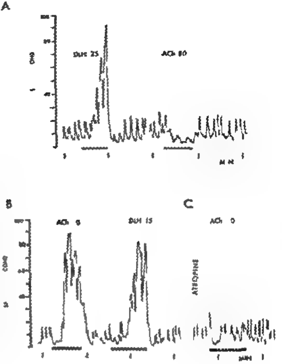

Fig 10 A — The frequency of firing of a thalamic neurone in response to DL-homocysteic acid (DLH 25 nA) and acetylcholine (ACh, 80 nA). Both ejections are indicated by the lower signal
B — The frequency of firing of another thalamic neurone evoked by acetylcholine (ACh 40 nA) and DL-homocysteic acid (DLH 15 nA)
C — Same cell as B but 7 minutes after a continuous current of 10 nA had been ejecting atropine from an other barrel of the 5 barrel micropipette

solution of 5 HT-creatinine sulphate In many cases however 5 HT depressed the spontaneous spindle activity of thalamic neurones an effect which had a latency of 20—30 sec when currents of 20—40 nA were used (Fig 9C) Recovery occurred within 30 sec of terminating the electrophoretic ejection Furthermore although the initial short latency synaptic response of thalamic neurones to peripheral or cortical stimulation remained unaltered by 5 HT the later burst responses were occasionally depressed and the intervening positive potentials reduced in magnitude Additional evidence for a mild depressant action of 5 HT was the finding on 18 cells that the frequencies of firing evoked by ACh or DL-homocysteic acid were reduced during the ejection of 5 HT In general the firing in response to ACh was more sensitive to this depression than that produced by the amino acid In some cases the depression of amino acid firing was not sustained during a 60—90 sec ejection of 5-HT but was replaced by a facilitation

Similar results were obtained with 3 hydroxytyramine and 4-HT Although no attempt was made to obtain accurate potencies of these agents as depressants the impression was obtained that there was very little difference between them and 5-HT It was noted however that the recovery of depression after the administration of 4 HT exceeded the time observed for 5-HT The depressant

action of all of these compounds was much less than that of γ amino n butyric acid which when ejected as a cation from solutions of pH 3—3 5 readily blocked both the synaptic and chemically evoked spikes of ventrobasal thalamic neurones

The pharmacology of synaptic firing

The types of synaptic response which were studied in these experiments included the short latency response elicited by stimulation of peripheral cutaneous nerves the repetitive burst response which followed this type of single shock stimulation short latency and repetitive burst responses which followed stimulation of the primary somatosensory cortex, and the spontaneous random and spindle discharges

The failure of DHβE and of atropine to effect these synaptic responses has already been discussed, and this finding suggests that none of the above types of synaptic excitation is cholinergic It must be realised however that despite the ability of these agents to block the excitation produced by electrophoretically administered ACh it is still possible that synaptically released ACh could remain effective since this would be applied in closer proximity to subsynaptic receptor sites and presumably in comparatively high concentration (see Dale and Gaddum 1930) The depressant action of 5 HT and some related substances has been discussed in the previous section

Mention was made in the previous paper (Andersen and Curtis 1964) that the spontaneous activity of many thalamic neurones was depressed during the period between the beginning of the ACh ejection and the onset of firing The magnitude of this effect depended somewhat upon the electrophoretic current, and upon the latency and effectiveness of the subsequent excitation In some cases particularly when ACh was a weak excitant there was complete suppression of spontaneous spindle activity (Fig 10A) In other cases the latency of excitation was so brief that any suppression of spontaneous activity was obscured In the case of the cell illustrated in Fig 10B there was a clear depression of spontaneous spindles by ACh but not by DL homocysteic acid This action of ACh was not due to the current used for electrophoretic ejection because such depression was not observed when even larger cationic currents were passed through adjacent NaCl containing barrels In some cases in which ACh depressed the burst responses evoked by peripheral or cortical stimulation the inter burst positive potentials were reduced in magnitude

An extensive pharmacological analysis of this depression was not carried out The excitant action of most of the compounds of Table I was often preceded by an early depression of spindles In several cells one of which is illustrated in Fig 10B and C although atropine blocked the excitant action of ACh the preliminary depression was apparently unaffected Similar observations were made using DHβE although the concomitant excitant action of this substance necessitated the use of very small electrophoretic currents

Discussion

The results of this and of the previous paper, permit discussion of three major questions the nature of the receptor with which ACh interacts whether these receptors are located post synaptically beneath excitatory synapses and lastly whether ACh is the natural transmitter operating at these sites

The subdivision of peripheral ACh receptors into muscarinic and nicotinic types depends upon the relative activities of certain cholinomimetic substances together with the actions of nicotine and atropine (see Dale 1914) It is unknown to what extent differences in the disposition of active sites within the receptor contribute to the pharmacological behaviour of these receptors Other factors including the ease with which substances can gain access to the sites and with the location and activity of cholinesterases may also be important Central ACh receptors are not easily classified into these two classical types (see Feldberg 1950) although it is only recently that cholinomimetic substances have been administered in sufficiently close proximity to central receptors to enable their nature to be determined Numerous experiments have established that the cholinoceptive receptors of spinal Renshaw cells are of the nicotinic type and bear some resemblance to the receptors of autonomic ganglia (Eccles *et al* 1954 1956 Curtis and Eccles 1958a b Curtis *et al* 1961 Curtis and Ryall 1964) In contrast the ACh receptors of cells located in the cerebral cortex are of a muscarinic type (Krnjevic and Phillis 1963b c) Thus nicotine has been reported to have no specific action upon cortical cells which were extremely sensitive to ACh muscarone muscarine and acetyl β methylcholine Furthermore the excitation of these neurones was prevented very effectively by atropine but not by dihydro-β erythroidine (Krnjevic and Phillis 1963c)

The ACh receptors within the thalamus are clearly of an intermediate type and are similar to those of lateral geniculate neurones (Curtis and Davis 1963) and neurones in the medullary reticular formation (Salmoiraghi and Steiner 1963) The slow onset and prolonged duration of the excitation produced by ACh is in marked contrast to the rapid and brief effect of this ester upon Renshaw cells (Andersen and Curtis 1964) but is very similar to the action upon cortical neurones (Krnjevic and Phillis 1963b) Carbamoylcholine was a more powerful excitant of thalamic neurones than ACh whereas dl muscarine nicotine and acetyl β methylcholine were less active The contrast between the actions of tetramethyl and tetraethylammonium upon thalamic and Renshaw cells has already been pointed out in the Results

The excitatory action of ACh was readily depressed by DHβE which in larger doses also excited thalamic neurones In this respect thalamic neurones resemble Renshaw cells see Curtis et al 1961 Although atropine depressed the ACh sensitivity of thalamic neurones the firing evoked by an excitant amino acid was also reduced by this substance Thus as with Renshaw cells

(Curtis and Phillis 1960; Curtis and Ryall 1964) the action of atropine could not be considered as very specific. It is possible however that this lack of specificity in the thalamus is more apparent than real. If the receptors with which acetylcholine interacts were further from the orifice of the micropipette than were the amino acid receptors (see Andersen and Curtis 1964) the concentration of electrophoretically ejected atropine would always be lower at the former sites than at the latter. Thus when the atropine concentration became adequate to block the ACh receptors, the higher concentrations near the orifices of the recording and ejecting micropipettes would affect the spike generating mechanism of the nearby membrane and lower the sensitivity of the neurone to an excitant amino acid. This difficulty in resolving the specificity of the action of atropine could have been overcome to some extent by administering atropine systemically so that the concentration around thalamic neurones would have been more uniform.

As a result of these pharmacological studies it may be concluded that the cholinoceptive sites within the ventrobasal thalamus are not as nicotinic as those of Renshaw cells and not as muscarinic as those of cortical neurones. The differences between the receptors upon thalamic and Renshaw cells provide one explanation of the different time course of excitation produced by the electrophoretic administration of ACh upon these neurones although other factors need also to be considered (Andersen and Curtis 1964).

With regard to the possible sites of action of ACh within the thalamus, the different susceptibilities of neurones within and near this area suggest that ACh receptors are related to synaptic processes. The finding that most ventrobasal thalamic relay neurones were fired by electrophoretically administered ACh whereas the depolarization of thalamic interneurones remained subthreshold (Andersen and Curtis 1964) and neurones in nearby nuclei were insensitive to this substance, is most readily explained by proposing that ACh is acting pre- or post-synaptically at the endings of a fibre system which normally operates upon thalamic neurones, particularly upon the relay cells, but also to a lesser extent upon the interneurones. These findings also render as extremely unlikely an explanation of the excitation by ACh in terms of either an indirect effect upon blood vessels, or of a direct action upon non-synaptic neuronal membrane such as occurs at sensory endings and non-myelinated mammalian C fibres (Gray 1959; Douglas and Ritchie 1962). It should be pointed out that the action of ACh on these latter structures is nicotinic in nature and is blocked by d-tubocurarine (Armett and Ritchie 1961; Ritchie and Armett 1963). It is also considered unlikely that the excitant action of ACh and other cholinomimetics upon thalamic neurones is due to a presynaptic effect which results in the release of an excitatory transmitter. Such an action of ACh has been postulated to account for certain aspects of the synaptic process at autonomic ganglia and at the neuromuscular junction (see Koelle 1963). There is, however, no evidence that such a presynaptic mode of action

releases transmitters other than ACh and the finding that ACh does not excite all central neurones suggests that if indeed the action of this substance is pre synaptic it nevertheless plays an essential role in the synaptic excitation of thalamic neurones Another possibility that cannot be fully excluded at the present time is that ACh is operating as a non synaptic modulator of neurone activity either released as a secretion by specialized cells or arising as a meta bolic by product (Curtis 1961 Toman 1963) In this case the responses of neurones would be altered only if the appropriate receptors were present

It is however reasonable to consider that the excitatory effect of ACh upon thalamic neurones is due to an interaction with post-synaptic receptor sites in which case ACh may be identical with or closely related to the ex citatory transmitter which is normally released at such synapses The effect of ACh upon a particular neurone will then depend upon the number of appropriate synapses In this respect it is necessary to modify conclusions drawn in an earlier paper where the excitatory action of ACh upon lateral geniculate neurones was described (Curtis and Davis 1963) The sensitivity of neurones in this portion of the thalamus is similar to that of interneurones in the ventrobasal complex and pharmacological studies showed that it was extremely unlikely that ACh was the transmitter released upon geniculate neurones by fibres of the optic tract It was concluded that the ACh sensitivity was probably associated with the presence of non optic synapses but the paucity of choline acetylase in this region together with differences between the action of ACh upon thalamic and Renshaw cells led to the conclusion that ACh was unlikely to be the normal transmitter operating at these non optic synapses It is now obvious that such a conclusion is unwarranted and the relatively weak action of ACh upon these cells suggests that if the excitatory action is indeed associated with synapses there are very few of the appropriate type of synapse Furthermore such synapses may have an origin common to those upon neurones in the ventrobasal complex of the thalamus

The above conclusion that the presence of cholinergic synapses account for the sensitivity of thalamic neurones to ACh is in accordance with the presence of ACh (MacIntosh 1941) choline acetylase (Feldberg and Vogt 1948 Zetler and Schlosser 1955 Hebb and Silver 1956) and cholinesterase (Burgen and Chipman 1951 1952 Koelle 1954 Gerebtzoff 1959) in this area of the nervous system It is unlikely that a substance structurally related to ACh is the trans mitter at these synapses since Ryall (1963) has recently demonstrated that the cholinomimetic material extracted from mammalian brain consists predominant ly of ACh The present pharmacological investigation however has failed to determine which afferent pathway to the thalamus is cholinergic It is clear that the medial lemniscus pathway is non cholinergic since DHβE and atropine which readily reduced the sensitivity of thalamic neurones to ACh did not influence the firing of these cells by impulses initiated in cutaneous sensory fibres The failure of i v administered DHβE and atropine to affect such

firing also suggests that the neurones of the dorsal column nuclei are non cholinoceptive In addition the present study indicates that the synaptic pathways associated with the spindle discharges of thalamic neurones, and with the repetitive burst responses elicited by peripheral or cortical stimulation are also unlikely to be cholinergic None of these forms of synaptic excitation was markedly enhanced by the administration of cholinesterase inhibitors beyond the slight increase associated with the excitation produced by these substances Although it is probable that this excitation was associated with direct interaction between these substances and cholinoceptive sites it is conceivable that both neostigmine and physostigmine merely prevented the destruction by cholinesterase of ACh which was released spontaneously from cholinergic endings Further investigations will be necessary to determine the origin of the cholinergic fibres which synapse upon neurones in the ventrobasal complex and in the lateral geniculate nucleus

Another problem which warrants further investigation is the depression of thalamic neurones by many cholinomimetics which eventually depolarize the same neurones One explanation of this phenomenon is that ACh excited nearby inhibitory neurones which in turn inhibited the cell under observation However the spike responses of such inhibitory neurones were not observed and, in addition no such depression was ever observed when DL homocysteic acid was ejected It is possible that the depression of thalamic neurones by cholinomimetics is associated with a non synaptic action upon the cellular membrane on the other hand ACh as well as being an excitatory transmitter may also be an inhibitory transmitter at other synapses upon these thalamic neurones In this respect the observation that the interburst positive potentials were reduced in amplitude by ACh may be significant since the electrophoretic ejection of an inhibitory transmitter would be expected to reduce the amplitude of inhibitory post synaptic potentials If ACh is an inhibitory transmitter such inhibitory synapses may be few in number but closer to the spike producing portion of the neurones Thus the depression resulting from the ejection of ACh would be of shorter latency but less powerful than the excitation The apparent depressant action of acetyl β methylcholine and n butyrylcholine upon lateral geniculate neurones (Curtis and Davis 1963) might have a similar origin

The weak depressant action of 5 HT upon neurones of the ventrobasal thalamus is in distinct contrast to the very specific action of this substance in blocking the excitation of lateral geniculate neurones by optic tract impulses (Curtis and Davis 1962) The difference between the action of 5 HT upon neurones in these two major thalamic relay nuclei is of some importance since it indicates that the transmitter released at the terminals of the optic tract is unlikely to be identical with that released by cutaneous afferent pathways which terminate in the ventrobasal complex Further investigation is necessary to establish whether the depression of the spontaneous spindle activity and late

burst responses of ventrobasal cells by 5 HT is a specific blocking effect at the particular excitatory synapses which are involved or is merely associated with an overall depression of neuronal responses produced by this substance The mode of action of 5 HT and related compounds has not been established but the observation that the excitation of thalamic neurones by ACh and excitant amino acids was also depressed raises the possibility that 5 HT could be an inhibitory transmitter or related to an inhibitory transmitter acting upon these cells On the other hand 5 HT may merely have a non specific depressant effect upon the neuronal membrane The results obtained with 5 HT are very similar to those reported by Krnjevic and Phillis (1963d) who studied cortical neurones although in this thalamic investigation a marked excitatory action of 5 HT was not observed presumably because sufficiently large electrophoretic currents were not used

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Acta physiol scand 1964 61 121—129

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Histochemical and Biochemical Observations on the Effect of Reserpine on Noradrenaline Storage in Vasoconstrictor Nerves

By

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Abstract

K FUXE and G SEDVALL. *Histochemical and biochemical observations on the effect of reserpine on noradrenaline storage in vasoconstrictor nerves* Acta physiol scand 1964 *61* 121—129 — Tissue sections of cat skeletal muscle were analysed for adrenergic fibres by a specific histochemical fluorescence technique Fibres with green fluorescence and varicosities typical of adrenergic fibres were seen almost exclusively surrounding the external muscular layer of the small arteries in the tissue No adrenergic nerve fibres could be detected after postganglionic sympathetic denervation of the muscles Reserpine (5 mg/kg i v) caused a complete disappearance of the fluorescence of the fibres within a few hours after administration Decentralization of the sympathetic nerves to the muscles prevented the fibres from losing fluorescence after reserpine An infusion of noradrenaline 24 hours after reserpine administration caused a reappearance of fluorescent nerve fibres of practically normal appearance A certain amount of noradrenaline could be detected by biochemical assay both in decentralized muscles 5 to 9 hours after reserpine administration and in muscles from reserpine treated animals in which noradrenaline had been infused. The data support the view that a small amount of noradrenaline can be retained in adrenergic nerves by mechanisms which are resistant to the effect of reserpine

A specific histochemical fluorescence method for the demonstration of catecholamines and 5 hydroxytryptamine in tissues has recently been developed (Falck *et al* 1962 Carlsson Falck and Hillarp 1962 Falck 1962) This method makes it possible to visualize adrenergic nerve fibres in tissues

In cat skeletal muscle noradrenaline is stored almost exclusively in adrenergic nerves (Sedvall 1964 a) The only adrenergic fibres that have been demonstrated in muscle are vasoconstrictor nerves (Folkow and Uvnas 1948) The histochemical fluorescence technique could be used to determine whether

there exists an adrenergic innervation of quantitative importance also to structures in skeletal muscle besides the blood vessels

High doses of reserpine deplete most tissue store of noradrenaline within a few hours after administration (Carlsson and Hillarp 1956 Bertler Carlsson and Rosengren 1956, Holzbauer and Vogt 1956) This action abolishes the effects of adrenergic nerve stimulation (Carlsson *et al* 1957 Burn and Rand 1958) Recently it was shown that decentralization of the sympathetic nerves to cat skeletal muscle prevented the rapid disappearance of a small noradrenaline fraction from the muscles after reserpine (Sedvall 1964 b) This finding would indicate the existence of different noradrenaline compartments in skeletal muscle *Thus it is of interest to examine the localization of the reserpine* resistant noradrenaline fraction by the histochemical method

In reserpinized animals the effects of adrenergic nerve stimulation can be restored after an infusion of noradrenaline (Burn and Rand 1960 Rovell and Sedvall 1961) The exact localization of the infused noradrenaline is unknown It is therefore of interest to examine with the histochemical method also muscles from reserpinized animals infused with noradrenaline

The present investigation was designed (1) to study the distribution of adrenergic fibres in muscle, (2) to localize the reserpine resistant noradrenaline fraction unmasked by decentralizing the adrenergic nerves before reserpine administration and (3) to study the localization of infused noradrenaline in skeletal muscle in reserpinized cats

Methods

General

The experiments were performed on cats (2 3– 3 4 kg) anesthetized with urethane (530–800 mg/kg i v) Control cats and cats in which sympathetic denervation had been performed earlier (see below) were killed by i v air immediately after anesthesia Different parts of the tibialis anterior and gastrocnemius muscles from both hind legs were removed immediately for histochemical and biochemical analyses In all animals the tibialis anterior and gastrocnemius muscles were both examined histochemically However as the noradrenaline stores of these muscles have about the same magnitud and the same sensitivity to reserpine (Sedvall 1964 a b) only one of them was analyzed biochemically (see Table I and II)

Postganglionic sympathetic denervation of the muscles in one hind limb of cats was performed seven days before killing the animal During operation these animals wer anesthetized with sodium pentobarbital (Nembutal Abbott 35 mg/kg i v) One of the sympathetic chains was reached from the anterior approach and removed from the level of L3 to S2 under aseptic conditions The animals were given 40 ml 0 9 saline s c and 1 ml Streptopenin (Kabi) after the operation The completeness of the operations was examined post mortem

In another series of experiments cats were anesthetized and the trachea cannulated Sympathetic decentralization of the muscles in one hind limb was performed by transecting one of the sympathetic chains at a level between L4 and L5 Reserpine (Serpasil Ciba 5 mg/kg) was then injected i v Rectal temperature was maintained at 36–37 C

by means of a heating lamp. After 5 to 9 hours the animals were killed and the homologous muscles from both hind limbs were immediately removed and prepared for histochemical and biochemical analyses.

In a third series of experiments cats were pretreated with reserpine (5 mg/kg s.c.). About 24 hours later the animals were anesthetized, tracheotomized and both sympathetic chains were transected as above. These animals were then prepared for recording of blood flow through the muscles of a hind limb in accordance with the method described by Lindgren 1958 (for details see Rosell and Sedvall 1961). One of the carotid arteries was cannulated and connected to a Statham pressure transducer P 23 AA for blood pressure recording. Rectal temperature was maintained at 36—37° C as above. Noradrenaline was then infused slowly into the femoral artery at a rate of about 1 μg/min through a side arm of the drop chamber. During infusion the blood flow through the muscles decreased by approx. 80 per cent. A total amount of 30 μg was infused. Only a slight increase in the general blood pressure was observed during infusion indicating that very small amounts of the infused material reached the general circulation and the other hind leg. After infusion the blood flow returned to the original level within about 10 min. A time interval of at least 30 min was allowed to pass after infusion before the animal was killed and the homologous muscles from both hind limbs removed for histochemical and biochemical analyses.

Histochemistry

In principle the method devised by Falck 1962 was used. Small pieces from different parts of the muscles were rapidly cut out and frozen in propane cooled by liquid nitrogen. After freezedrying *in vacuo* at −35° C for 5 days the tissue specimens were treated with formaldehyde gas at 80° C for one hour. During this treatment primary catecholamines and 5-hydroxytryptamine condense — without diffusion — with formaldehyde to intensely fluorescent products. The specimens were then embedded in paraffin *in vacuo*. Sections were made 10 μ thick and mounted in Entellan (Merck) after removal of the paraffin (cf. Falck 1962). The sections were examined in light from an Osram HBO 200 high pressure mercury lamp equipped with Schott BG filters (3 to 5 mm). A fluorescence microscope was used with darkfield condensors (Zeiss) and a Kodak Wratten filter 15 or a Schott OG4 (1 mm) in the tube. Some of the sections were stained with hematoxyline-eosine and Weigerts-elastin after examination in the fluorescence microscope to localize the fluorescent nerves.

Biochemistry

The noradrenaline content of the muscles was determined fluorimetrically essentially in accordance with Bertler, Carlsson and Rosengren 1958. Details of the method used are given elsewhere (Sedvall 1964 a). Throughout this paper noradrenaline is expressed in terms of free base as nanograms (10^{-9} g) per g wet weight of muscle.

Results

In muscles from 5 normal cats a sparse amount of green to yellow green fluorescent fibres was observed (Fig. 1). Varicosities were seen in some of the fibres. In the sections examined almost all the fluorescent nerve fibres were located in close proximity to small arteries or arterioles (Fig. 2 and 3). The innervation had the appearance of a loose network of fluorescent fibres surrounding the external muscular layer of the small arteries. In no case were fibres seen penetrating down into the muscular layers of the blood vessels

Fig 1 *M tibialis anterior* of cat Cross section Adrenergic nerves are present around the external muscular layer of the small arteries one of which (A) is cut transversely while the others are cut longitudinally (B) Fluorescence microphotograph Magnification 165 ×

Fig 2 *M tibialis anterior* of cat Cross section Adrenergic nerves are present surrounding the external muscular layer of a small artery which is cut transversely at one place (A) and longitudinally at another (B) Fluorescence microphotograph Magnification 165 ×

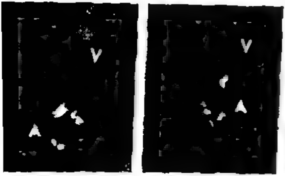

Fig 3 Left *M tibialis anterior* of cat Cross section Adrenergic nerves are present surrounding the external muscular layer of the small artery (A) The accompanying vein (B) receives no adrenergic nerves Fluorescence microphotograph Magnification 460 ×
Right *M tibialis anterior* of a cat pretreated with reserpine (5 mg/kg s c 24 hours before the experiment) and killed 30 min after an infusion of noradrenaline Adrenergic nerves are present around the external muscular layer of the small artery (A) in the same way as in normal animals The accompanying vein (B) receives no adrenergic fibres Fluorescence microphotograph Magnification 460 ×

Table I Fluorescence intensity of adrenergic fibres and noradrenaline content (NA ng/g) of innervated and sympathetically decentralized skeletal muscle after reserpine (5 mg/kg i.v.)

Animal no	Weight (kg)	Hours after reserpine	Control side Fluorescent fibres	NA	Decentr side Fluorescent fibres	NA
1	3.1	5	–	5	++	37
2	3.4	5	–	3	+	18
3	3.2	9	–	0	+	15
4	2.8	5	+	20	++	58
5	3.0	8	–	1	+	13

++ Fibres with normal fluorescence
+ Fibres showing a small decrease in fluorescence intensity
– No fluorescent fibres

In cats no 1 to 4 the tibialis anterior muscles were analyzed In cat no 5 the gastrocnemius muscles were analyzed

Fluorescent nerve fibres to the small veins were absent or scarce In sections where a small artery could be seen together with the accompanying vein the artery was surrounded in most cases by two or three nerve bundles whereas fluorescent fibres could not be seen around the vein except in a few isolated sections No localized pattern of innervation could be observed in any section of the vascular bed In 4 out of about 500 sections single fibres were seen between the muscle cells without any established connection with the blood vessels *The fluorescent fibres which appeared to be extravascular constituted at most* one per cent of the total amount in the muscles The same general innervation pattern was found in *M tibialis anterior* and *M gastrocnemius*

The effect of postganglionic sympathetic denervation of the muscles was studied in 3 cats No fluorescent fibres could be detected in the denervated muscles 7 days after operation The fluorescent fibres around the blood vessels in the homologous control muscles were of normal appearance

The acute effect of reserpine on the fluorescence of the nerve fibres and the noradrenaline content of innervated and sympathetically decentralized muscles was studied in 5 cats (Table I) In the decentralized muscles fluorescent fibres of practically normal appearance could be seen 5 to 9 hours after reserpine There was no significant reduction in the total amount of fibres The only *difference as compared with fibres in normal muscles was a small* but definite decrease in the fluorescence intensity of the fibres in 3 of the animals The noradrenaline content was decreased to about 30 per cent of the normal The noradrenaline content in normal cat muscles is about 100 ng/g (Sedvall 1964 a) No fluorescent fibres could be detected in the innervated muscles in 4 of the 5 cats The noradrenaline amount in these muscles was less than 5 per cent of the normal content In cat no 4 20 ng/g of noradrenaline was *still* present in the

Table II Fluorescence intensity[1] of adrenergic fibres and noradrenaline content[2] (NA ng/g) in skeletal muscle from reserpinized cats after an infusion of noradrenaline

Animal no	Weight (kg)	Minutes after infusion	Control side Fluorescent fibres	NA	Infused side Fluorescent fibres	NA
6	3.0	30	—	0	+	8
7	3.0	40	—	0	+	13
8	2.3	30	—	0	++	20
9	3.4	30	—	2	++	22

[1] ++ Fibres with normal fluorescence intensity
+ Fibres showing a small decrease in the fluorescence intensity
— No fluorescent fibres

[2] In all animals the gastrocnemius muscles were analyzed

innervated muscles Nerve fibres with a diminished fluorescence could also be detected in these muscles In the decentralized muscles from this animal however, there was a noradrenaline content of 58 ng/g and a much more intense fluorescence in the fibres It is probable that cat no 4 normally had an unusually high noradrenaline content in the muscles There is a wide range in the noradrenaline amount in muscles from different cats (Sedvall 1963 a)

Noradrenaline was infused i a into one hind leg of 4 cats pretreated with . Muscles from both hind legs were analysed 30 to 40 min after infusion The results can be seen in Fig 3 (right) and Table II In the muscles from the non infused control leg no fluorescent fibres and practically no noradrenaline could be detected In the muscles infused with noradrenaline on the other hand fluorescent fibres had appeared that were similar in both morphology and frequency of occurrence to the fluorescent fibres in normal muscle However a small but significant decrease in fluorescence intensity in comparison with normal fibres was observed in the fibres of 2 of the cats The muscles infused with noradrenaline contained about 20 per cent of the normal noradrenaline content

The faint background fluorescence seen in the sections was not affected by postganglionic sympathetic denervation or reserpine treatment and had approximately the same degree of intensity also without formaldehyde treatment (cf Falck 1962)

Discussion

The high specificity and sensitivity of the histochemical method used in the present investigation for the visualization of adrenergic fibres has recently been established in investigations by Falck et al 1962 Corrodi Falck and Hillarp 1962 Falck and Hillarp 1962 and Falck 1962 The green to yellow

green fluorescence as seen in the fibres in the present study can be produced by noradrenaline and dopamine However as the dopamine level in normal skeletal muscle is very low (Leduc 1961 Sedvall 1963, unpublished experiments), the fluorescence of the fibres was probably produced predominantly by noradrenaline The disappearance of the fluorescence of the fibres in muscle after sympathetic postganglionic denervation or reserpine treatment as found here agrees with the findings of the above authors on other organs and provides further evidence that the fibres represent adrenergic nerves The high sensitivity of the method is evident from the finding that the noradrenaline content of the muscles could be reduced to about 10 per cent of the normal before the nerve fibres lost their fluorescence

The scanty supply of adrenergic fibres in skeletal muscle is in good agreement with the low noradrenaline content in this tissue (Euler 1954 Sedvall 1964 a) Practically all the fluorescent nerve fibres were found in relation to the arteries Roughly one per cent of the fibres — having no apparent connection with the blood vessels — were seen between striated muscle bundles The vasoconstrictor fibres thus seem to constitute the predominant part of the adrenergic innervation of skeletal muscle The total noradrenaline content of cat skeletal muscle would thus be a good measure of the noradrenaline amount in the vasoconstrictor nerves

The adrenergic innervation of the blood vessels in skeletal muscle seems to be localized predominantly on the resistance (i e arterial) side of the vascular bed Practically no adrenergic fibres were found to innervate the veins The vasoconstrictor fibres were running along the blood vessels in close contact with the external muscular layer The fibres did not penetrate into the layer between the smooth muscle cells It is thus improbable that every cell in the muscular layer could be reached by adrenergic nerve terminals The transmission process could conceivably be mediated to the noninnervated smooth muscle cells by diffusion of the transmitter (Rosenblueth 1950) or by intermuscular spread of excitation (Roddie 1962) The finer organization of the synaptic contact sites cannot be ascertained with the present method Thus it is unknown whether noradrenaline is released only at the endings of the fibres or at several places along the terminal parts of the fibres

It was recently found that decentralization of the vasoconstrictor nerves to skeletal muscle prevented the disappearance of the physiological response to electrical stimulation of these nerves after reserpine (Rosell and Sedvall 1962) Noradrenaline analysis of the muscles under similar conditions revealed that a small noradrenaline amount in sympathetically decentralized skeletal muscle was resistant to the direct noradrenaline releasing effect of reserpine Sedvall 1964 b) In innervated muscles this small fraction was rapidly released after reserpine probably by the continuous impulse discharge in the adrenergic nerves In the absence of nerve stimuli the reserpine resistant fraction disappeared slowly over a period of more than 24 hours The result indicated that

noradrenaline in skeletal muscle is stored in two different compartments (Sedvall 1964 b) The present histochemical observations support these earlier findings and strongly suggest that the small reserpine resistant noradrenaline fraction is localized in the vasoconstrictor nerve endings in a manner similar to the normal distribution of the transmitter The resistant fraction does not seem to be present in only a few of the fibres — approximately the normal number of fluorescent fibres was observed in the sections The larger reserpine sensitive fraction seems likewise to be present along the whole length of the normal fibres as a generally reduced fluorescence intensity of the decentralized fibres after reserpine was found in most of the animals The present results thus indicate that the two noradrenaline fractions in skeletal muscle are both stored along the terminal parts of the vasoconstrictor neuron Several workers have presented evidence for the existence of different pools of noradrenaline in sympathetic tissues (Hillarp 1960 Euler and Lishajko 1961, Stjarne 1961, Trendelenburg 1961 Potter *et al* 1962 and Kopin and Gordon 1962) However information on the two suggested noradrenaline compartments in the vasoconstrictor nerves is still too meagre to permit any suggestions as to whether they are identical with any of the compartments suggested by the above mentioned authors

The reappearance of the fluorescent fibres in muscles from reserpinized cats after an infusion of noradrenaline is interesting in the light of the findings of Burn and Rand (1960) and Rosell and Sedvall (1961) These authors found that an infusion of noradrenaline could to a large extent restore effects of adrenergic ve stimulation in reserpinized animals After an infusion of noradrenaline n reserpinized cats a small amount of noradrenaline is furthermore retained in the muscles — in the absence of nerve stimuli for at least two hours after infusion There is only a small noradrenaline retention in sympathetically denervated muscles under similar conditions (Hertting *et al* 1961 Sedvall and Thorson 1964) There is thus physiological histochemical and biochemical evidence that the adrenergic nerves in skeletal muscle can take up exogenous noradrenaline and to some extent store it in spite of the action of reserpine It could be argued that by 24 hours after the injection the action of reserpine will have had time to subside However, another high dose of reserpine injected immediately before the infusion of noradrenaline does not abolish the restoration of the adrenergic nerve stimulatory effects (Rosell and Sedvall 1961 unpublished observations)

The endogenous reserpine resistant noradrenaline fraction and the exogenous infused noradrenaline fraction are similar in respect of magnitude localization in the adrenergic nerve terminals and sensitivity to nerve impulses However the rapid disappearance of the exogenous fraction after infusion (Sedvall and Thorson 1964) makes it uncertain whether the two fractions are retained by the same mechanism

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Localized Slow Wave Activity in the Somatosensory Cortex of the Cat

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Abstract

ANDERSSON S A and E R WOLPOW *Localized slow wave activity in the somatosensory cortex of the cat* Acta physiol scand 1964 *61* 130—140 Depth recordings with microelectrodes have revealed that transection of the specific projection pathways in the spinal cord induces regular slow waves with a frequency of 8—12 waves per second in the parts of the somatosensory areas SI and SII corresponding to the projection from below the level of the lesion *Surface recordings showed an increased spindle activity in the same areas* The slow wave activity occurs at various depths of Nembutal anesthesia There is evidence that the appearance of slow waves after interruption of the specific pathways is a general phenomenon in the specific projection cortical areas

It has been established with anatomical as well as physiological methods that certain well defined areas of the cerebral cortex receive short latency projections from specific afferent systems In the somatosensory areas I and II the face and body surfaces are represented in an orderly topographical fashion (Woolsey 1947 Berman 1961) The spinal pathways mediating this projection consist of the dorsal columns and a pathway ascending in the dorso medial part of the lateral funicle first described by Morin (1955) Both these pathways project upon the contralateral areas SI and SII as is evident from the cortical potentials evoked by light adequate stimuli applied to the skin or stimulation of dissected peripheral nerves (Mark and Steiner 1958, Norrsell and Voorhoeve 1962) and from single unit analysis of the cells in SII (Andersson 1962a) and SI (Andersson and Norrsell unpublished observations) After transection of the dorsal columns and the spino cervical tract ascending in the dorso medial part of the lateral funicle cortical potentials of short latency or activation of single cells can no longer be obtained in response to light adequate peripheral stimuli or electrical stimuli applied to the skin of a cat anesthetized with Nembutal In this type of preparation electrical stimulation of the dissected nerves gives only a small and inconstant cortical response of short latency

(Norrsell and Wolpow 1964) Thus it can be expected that an interruption of the dorsal columns and of the spino-cervical tract eliminates the main part of the afferent influx to the cortical cells in SI and SII which have topographically arranged short latency connections from the periphery via the spinal cord In a preliminary report it has been shown that such a lesion results in a change in the slow wave activity in SI and SII namely high voltage slow waves with a frequency of 8—12 per second which appear in projection areas with peripheral innervation fields situated below the level of the lesion (Andersson 1962b)

Thus far no systematical studies have been made of the EEG activity in the the specific projection areas of the somatosensory cortex after transection of the short latency projection pathways The finding that the spinal pathways projecting with short latency are located in the dorsal part of the spinal cord has provided the opportunity of analyzing the effects of selective lesions of these pathways in the somatosensory cortex as well as in other cortical areas

Methods

The experiments were performed on adult cats Ether anesthesia was used during the operations and the animals were afterwards kept on pentobarbitone sodium (Nembutal Abbot) in sufficient doses to prevent spontaneous movements To prevent reflex movements a neuromuscular blocking agent gallamonium triethiodide (Flaxedil May & Baker Ltd) was given in many experiments and the animals were then respirated artificially with a pump The rectal temperature of the animal was kept as close to 38 C as possible with the aid of a heating lamp

After laminectomy the spinal lesions were made either at the level of C_3—C or in the lower thoracic cord with the aid of fine forceps and a binocular dissecting microscope The lesion included the dorsal columns and the dorsal part of the lateral funiculus bilaterally All lesions were controlled histologically The part of the spinal cord containing the lesion was fixed in 10 formaline embedded in paraffin and sectioned serially at 50μ

In most experiments the cortical surface was exposed only over the somatosensory cortex and the electrical activity was recorded with a micropipette filled with 4 M NaCl solution and mounted in a closed chamber (for details see Andersson 1962 a) The potential differences between the electrode tip and a second lead attached to the scalp were led through a cathode follow s to a RC amplifier (Grass 15 and displayed on a cathode ray oscilloscope (Tektronix 502 from which photographic records could be taken on running film In many experiments an inkwriter (Mingograph 24 B was coupled in parallel with th oscilloscope The frequency response curve of th amplifier with open filtres has its 1 2 amplitude at 0 1 Hz and 76 kHz In some experiment the cortical surface was widely exposed the dura was removed and the cortex was covered with a pool of mineral oil at 38 C The cortical activity was then recorded with silver silverchlorid ele tr des in contact with the surface

The cortical evoked potentials o activity of single cell were induced by peripheral adequate or electrical stimulation Jets of air a light brush pencil [illegible] a blunt probe were used [illegible] light adequate stimuli Pinching with a toothed pa of forceps or firm pressure were regarded as strong stimuli Short electrical stimuli were delivered to the skin with a pa of needles ins lated except on the t p

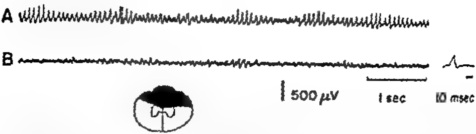


Fig 1 Oscillographic macroelectrode recording of the spontaneous cortical activity at a depth of 1 000µ below the cortical surface Extent of spinal cord lesion at Th_1 is indicated by black area in drawing below the records Record A shows activity typical for the denervated cortex (hindlimb area of SI) Record B shows the activity in the innervated cortex (forelimb area of SI) Inset to the right are records of the effect of short tactile stimuli to the contralateral hind paw (A) and to the contralateral forepaw (B) No evoked potentials are obtained in the denervated cortex. Negativity upwards

Nomenclature *The short latency spinal pathway ascending outside the dorsal columns* in the dorso-medial part of the lateral funicle and relaying in the lateral cervical nucleus will be referred to as the *spino-cervical* tract The dorsal columns and the spino-cervical tract are denoted *specific projection pathways* The parts of cortical areas SI and SII corresponding to the projection from peripheral fields below the level of a lesion including the dorsal columns and the contralateral spino-cervical tract are referred to as the *denervated cortex* The parts of the cortex with all the afferent pathways intact are termed the *innervated cortex*

Results

Slow wave activity in the denervated cortex

a *General characteristics* The most striking feature of the denervated cortex is the appearance of regular slow high voltage waves with a frequency of 8—12 waves per second (Fig 1A) This slow wave activity is regularly obtained after interruption of the dorsal columns and the spino cervical tract contra lateral to the investigated hemisphere The slow wave activity is clearly seen only in microelectrode recordings at some depth in the cortex Only occasionally regular slow waves different from the activity in the innervated cortex were observed in surface recordings from the denervated cortex In depth recordings the wave activity in the denervated cortex is completely different from that obtained under the same conditions of the animal in the innervated cortex Depending upon the depth of anesthesia the innervated cortex may show different degrees of wave activity but the waves do not have a continuous and regular pattern similar to that of the denervated cortex Under the conditions of the present experiments the EEG of the innervated cortex usually shows a low voltage high frequency (desynchronized) activity occasionally interrupted by spindles (Fig 1B)

The amplitude of the slow waves in the denervated cortex showed spontaneous variations with waxing and waning in a similar way as the spindle

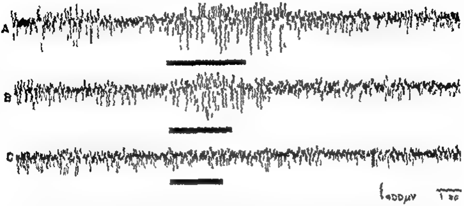


Fig 2 Microelectrode recordings (inkwriter) from a depth of 800 μ below the cortical surface in the denervated cortex (forelimb area of SI) Record A shows the spontaneous wave activity and the effect of pressure on the ipsilateral hindpaw immediately after transection of the specific projection pathways at C. Infiltration of the lesion with a local anesthetic did not change the slow wave activity or the effect of pressure on the hindlimb (B) Infiltration of the ventral quadrants with local anesthetic and subsequent spinalization abolish the effect of stimulation but does not change the slow wave activity (C) Black bars indicate duration of stimulation Negativity downwards

activity in the innervated cortex These amplitude changes were more frequent in the denervated than in the innervated cortex This was particularly evident in simultaneous surface recordings from the innervated and the denervated cortex

In some experiments the specific projection pathways were transected during continuous microelectrode recording of the EEG in regions of SI or SII corresponding to the projection from fields below the level of the intended lesion The slow continuous wave activity typical of the denervated cortex appeared as soon as the dorsal columns and the contralateral spino-cervical tract were interrupted (Fig 2 A) Infiltration of the spinal cord lesion with a local anesthetic did not change the slow waves (Fig 2 B) The local application of the anesthetic did *not* interfere with the transmission in the ventral spinal pathways since strong stimuli to peripheral areas below the level of the lesion gave a changed EEG pattern The EEG changes induced by such stimulation will be described in a following paper (Andersson Norrsell and Wolpow 1964) The slow wave activity was neither changed when the ventral cord was blocked by injection of local anesthetics into the cord nor when the spinal cord was completely transected Fig 2 C)

b *Effects of different depths of anesthesia* The regular slow wave activity in the denervated cortex is present under various depths of Nembutal anesthesia This is illustrated in Fig 3 The slow wave activity was recorded in the hind limb area of SI at a depth of 1 000 μ in a cat with the specific projection pathways transected at C Fig 3A shows the wave activity before additional

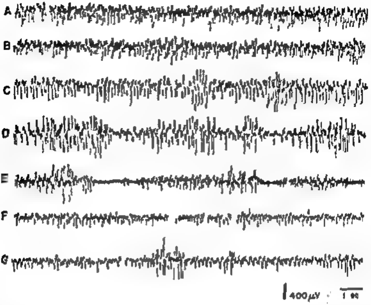


Fig 3 Slow wave activity recorded (inkwriter) through a microelectrode at a depth of 1 000 µ in the denervated cortex Effect of changes in the depth of anesthesia Record A shows the activity before any additional amount of Nembutal was given Records B—E were taken with 5 min intervals and 2 min before each record was taken 10 mg of Nembutal were given i v Record F taken 90 min after record E Record G shows the effect of an additional dose of 10 mg of Nembutal. Negativity downwards.

doses of Nembutal were given The animal did not show any spontaneous movements but reacted to pinching with flexion reflexes and dilatation of the pupils The successive records B—E show the effect of a total of 40 mg of Nembutal i v in doses of 10 mg at 5 min intervals After only 10 mg of Nembutal the amplitude of the waves increased and the frequency decreased (B) This is more pronounced when 20 mg had been given (C) The amplitude of the waves shows spindle like changes and after 30 mg (D) these large amplitude spindle waves dominate the record After 40 mg Nembutal the spindles become longlasting and the interspindle periods have low voltage activity (E) The animal was then allowed to recover from the anesthesia and the EEG activity was recorded at intervals of 5 min The activity recovered in reversed order Record F is taken 90 minutes after the last injection of Nembutal The continuous regular slow waves have returned and are similar to those in record B The last record (G) shows the effect when 10 mg Nembutal is given to the animal again The slow waves show larger amplitude and a lower frequency together with increased spindling similar to the activity in record C.

c *Areas showing regular slow wave activity* The parts of the somatosensory cortex showing the high voltage regular slow wave activity described above are confined to the areas in SI and SII corresponding to the projection from periph

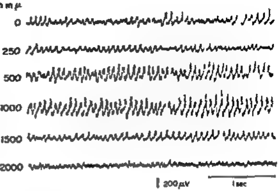
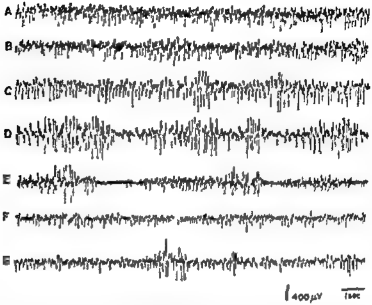


Fig 4 Oscillographic recordings of the spontaneous activity at different depths below the cortical surface in one microelectrode penetration in the denervated cortex The penetration was made in the forelimb area after interruption of the specific projection pathways at C_4 No short latency potentials were evoked by light tactile stimuli to the contralateral forepaw Negativity upwards

eral fields below the level of the transection of the dorsal columns and the contralateral spino-cervical tract in the spinal cord Thus interruption of the dorsal columns and the spino-cervical tract at the level of C_{3-4} gives an appearance of slow high voltage regular waves in those parts of SI and SII corresponding to the projection from the body and the limbs below the level of the lesion However the face areas of SI and SII show no significant change in the EEG activity If a similar spinal lesion is made at low thoracic level the appearance of slow regular waves is confined to the areas in SI and SII corresponding to the specific projection from the hindlimbs

Microelectrode penetrations in the somatosensory cortex indicate that the appearance of slow waves is strictly confined to the denervated parts of areas SI and SII In one type of experiment the cortical projection areas of the contralateral hindlimb and forelimb were mapped with surface recordings of the positive negative wave potentials obtained in response to a brief tactile stimulus applied to the skin After transection of the specific projection pathways either at low thoracic or at high cervical level this mapping procedure was repeated It was regularly found that the lesion abolished the cortical wave potentials in response to adequate stimuli to the parts of the body below the level of the lesion but there was no change in the response obtained by stimulation of peripheral regions with intact specific projection pathways As the next step in these experiments microelectrode penetrations were made in the regions of SI and SII mapped as the hindlimb forelimb and face areas It was found consistently that the penetrations made in cortical areas with intact specific projection pathways (forelimb and/or face regions) showed an EEG activity with small fast wave activity occasionally interrupted by spindles (Fig 1 B) Microelectrode penetrations in projection areas corresponding to the parts of the body below the level of the spinal lesion (hindlimb and/or forelimb areas) gave EEG activity with slow high voltage waves typical for the denervated cortex (Fig 1 A)



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doses of Nembutal were given The animal did not show any spontaneous movements but reacted to pinching with flexion reflexes and dilatation of the pupils The successive records B—E show the effect of a total of 40 mg of Nembutal i.v in doses of 10 mg at 5 min intervals After only 10 mg of Nembutal the amplitude of the waves increased and the frequency decreased (B) This is more pronounced when 20 mg had been given (C) The amplitude of the waves shows spindle like changes and after 30 mg (D) these large amplitude spindle waves dominate the record After 40 mg Nembutal the spindles become longlasting and the interspindle periods have low voltage activity (E) The animal was then allowed to recover from the anesthesia and the EEG activity was recorded at intervals of 5 min. The activity recovered in reversed order Record F is taken 90 minutes after the last injection of Nembutal The continuous regular slow waves have returned and are similar to those in record B The last record (G) shows the effect when 10 mg Nembutal is given to the animal again The slow waves show larger amplitude and a lower frequency together with increased spindling similar to the activity in record C

c. *Areas showing regular slow wave activity* The parts of the somatosensory cortex showing the high voltage regular slow wave activity described above are confined to the areas in SI and SII corresponding to the projection from periph

deeper into the cortex the amplitude increased to a maximum at 500—1 000 μ. At a depth of 1,500 μ the amplitude is reduced and at 1 800—2 000 μ no slow waves could be detected. However in some penetrations the slow wave activity was found at depths more than 2,000 μ below the surface. A considerable slow wave activity can also appear in the innervated cortex but the waves are then much more irregular in both amplitude and frequency (Fig. 5).

II. *Activity of single cells in the denervated somatosensory cortex*

The unit activity recorded with a microelectrode in the denervated somatosensory cortex is quite different from that found in the innervated cortex. In the denervated cortex, i. e. after transection of the dorsal columns and the spino-cervical tract, only few units were spontaneously active. Most units observed showed discharges of injury type, probably due to damage of the cell caused by the electrode since the recorded potentials were initially positive and occurred in bursts with decreasing amplitude. The spontaneously active cells had either a low rate of random discharge or were active in association with the slow high voltage waves. Practically all the cells of the latter type discharged in bursts on the most negative part of the waves. Some of the cells fired on the top of every wave regardless of its amplitude. Other units discharged more sporadically in connection with waves. These units are usually fired only on the largest waves either when the amplitude increased in a spindlelike manner or if the size of the waves increased due to peripheral stimulation (Andersson, Norrsell and Wolpow 1964). In the denervated cortex no cells could be activated after short latency by light peripheral stimuli.

Discussion

The microelectrode recordings show that a regular slow wave activity with the frequency of 8—12 waves per second appears in the somatosensory cortex after a transection in the spinal cord of the specific projection pathways, i. e. the dorsal columns and the spino-cervical tract. The regular slow waves were found only below the cortical surface having a maximum amplitude at a depth of 500—1 000 μ. This is also the depth where the short latency potential evoked by a peripheral stimulus has its maximum amplitude when recorded through a microelectrode in the intact cat (Li, Cullen and Jasper 1956). Intracellular recordings in the denervated cortex showed cells with large membrane potential fluctuations (Andersson, Lundberg and Wolpow 1964) and there are good reasons to believe that the slow waves described here constitute the electrical field from large synchronous membrane potential changes in many cells.

The slow wave activity appears only in the cortical areas in SI and SII corresponding to the projection areas from parts of the body below the level of the spinal lesion. Although the EEG activity shows different degrees of slow wave activity with irregular frequency and spindles depending upon the depth

of anesthesia the difference in activity between the innervated cortex and the *denervated cortex is very marked* This difference could be observed both in different penetrations of the denervated and innervated cortex in the same experiment and in the same penetration on the borderline between innervated and denervated cortex The difference between innervated and denervated cortex has been controlled in all the experiments in order to make sure that the recorded slow wave activity in the somatosensory cortex was a local phenomenon and *not a generalized slow wave activity due to the anesthesia or the* general condition of the animal Recordings at the cortical surface either with the microelectrode in contact with the pial membrane or with the silver silver chloride electrode resting on the cortical surface did not usually show a continuous regular slow wave activity At this level the EEG was characterized by spindle bursts and low voltage activity in the interspindle periods in both the denervated and the innervated cortex However the spindle bursts appeared more frequently in the denervated cortex Similar observations were made by Claes (1939) Roger Rossi and Zirondoli (1956) and Arduini and Hirao (1959) in the visual cortex after deafferentation of the eyes These authors found an increase in spindling with desynchronized interspindle periods in the projection area Roger et al (1956) found similar changes in the auditory cortex after interruption of the acoustic nerves *An increase in the spindle activity seems thus to be a common phenomenon for the specific projection cortical areas after elimination of the afferent inflow from the corresponding receptor systems* although further experiments with depth recording in the visual and auditory cortices are required in order to permit a decisive comparison with the present findings

The importance of sensory impulses for desynchronization of the EEG was pointed out by Bremer (1935) who found a sleeping pattern with generalized high voltage slow waves in the EEG in the cerveau isolé preparation Bremer assumed that the cortical synchronization was due to deprivation of sensory inflow to the cortex This synchronization is even more pronounced after additional visual denervation (Bizzi and Spencer 1962) Moruzzi and Magoun (1949) showed that generalized cortical desynchronization could be obtained by electrical stimulation in the reticular formation and postulated that the alert EEG pattern was *due to a tonic activity in the activating ascending* reticular system and that a reduction in this tonic activity in the ascending reticular system will cause a cortical synchronization In many later investigations the generalized desynchronizing effects from different parts of the ascending activating system have been studied and confirmed (Rossi and Zanchetti 1957) It is also well established that sensory stimulation gives rise to EEG desynchronization (arousal) and there is good evidence that this effect is mediated via the ascending activating system (Pompeiano and Swett 1962)

The main problem raised by the present findings is whether the suppression of slow waves in the innervated projection areas is due to the tonic activity in the ascending activating system In all likelihood it is not so the absence of

slow waves being due to a different mechanism In the present experimental conditions generalized cortical activation could easily be obtained in the denervated as well as in the innervated cortex via ventral pathways in response to strong stimuli These effects are at least partially mediated via a pathway ascending in the ventral spinal cord with termination in the brain stem described by Lundberg and Oscarsson (1962) Stimulation below the level of the interruption of the specific projection pathways elicits a much stronger generalized cortical activation than stimulation above the level of the lesion (Andersson Norrsell and Wolpow 1964) However in a preparation with transection of the spinal cord except for the dorsal columns no generalized cortical effects can be elicited in response to peripheral stimulation below the lesion although light stimuli in the same peripheral fields give a large evoked potential in the cortical projection areas and a localized desynchronization (Andersson unpublished) This indicates that the specific projection pathways are not primarily involved in generalized cortical arousal This is further supported by the scarcity of anatomical connections between the medial lemniscus and the reticular formation (Matzke 1951 Rossi and Brodal 1957 Scheibel and Scheibel 1958 Bowsher 1958)

Thus there is strong evidence indicating that the localized slow wave activity induced by transection of the specific projection system is not due to a decrease in the tonic activity in the ascending activating system The problem of how the impulses in the specific projection system can prevent the appearance of the slow waves in an area strictly limited to the field of its cortical projection will be discussed in a following paper (Andersson Norrsell and Wolpow 1964)

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From the Department of Physiology University of Goteborg Sweden

Intracellular Slow Wave Potentials in the Somatosensory Area of Cat

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Abstract

Andersson S A, A Lundberg and E R Wolpow *Intracellular slow wave potentials in the somatosensory area of cat* Acta physiol scand. 1964 *61* 141—143 Intracellular recordings from cells in the first somatosensory area of the cerebral cortex have been made in cats in which the spinal pathways projecting to this area have been transected There are large rhythmic membrane potential changes which correspond to the extracellularly recorded slow waves

Experiments with intracellular recordings from cortical cells have given valuable information regarding the properties of cortical neurones and the synaptic actions evoked in them (Phillips 1959 Li 1961 Kandel Spencer and Brinley 1961) However less attention has been given to the origin of the slow waves that can be recorded from the surface and from the depth of the cortex It has recently been shown that in the somatic sensory areas very large slow wave activity is induced by transection of the spinal pathways projecting to these areas (Andersson 1962 Andersson and Wolpow 1964) An excellent opportunity is hereby offered to investigate in more detail the cellular mechanism of the slow wave activity In the present preliminary report it will be shown that there are corresponding membrane potential changes in cortical cells

Methods

Experiments were performed on cats lightly anesthetized with pentobarbitone sodium The skull was opened to expose the first somatosensory area. Laminectomy was made in the mid thoracic region and the dorsal columns and the dorsal part of the lateral funiculi transected Conventional glass capillary microelectrodes filled with 3 M KCl solution with a resistance of approximately 5 MΩ were used for recording A closed chamber system was used to minimize movements A chamber was attached to the scalp

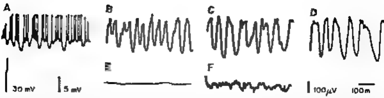

Fig 1 Intracellular recording from a cell in the first somatosensory area The spike discharges in A were taken at the lower amplification a few seconds after the impalement The consecutive records B—D were taken about 1 min afterwards and amplification is indicated by the 5 mV calibration below record A Record E and F taken after withdrawal of the microelectrode to an extracellular position E at the amplification used in B—D and F at the higher amplification shown below D

filled with 0 9 % saline and covered with a glass disc through which the microelectrode was inserted The manipulator carrying the electrode permitted movements of less than 1 microne The time constant of the recording amplifier was 0 8 sec and a DC amplifier in parallel was used to measure resting membrane potentials

Results

Intracellular recordings were made from 19 cells in the hindlimb area of SI after transection of the spinal specific projection pathways No intracellular recordings were made in other cortical areas The mean value of the resting membrane potential was 35 mV In 7 of the cells rhythmical membrane potential changes were observed as illustrated in Fig 1 After impalement the cell initially discharged violently in bursts as seen in record A The discharge ceased after 30 sec and there remained slow potential fluctuations which are shown at higher amplification in three consecutive records (B—D) The membrane potential was 40 mV when records B—D were taken The amplitude of the potential fluctuations in B—D often exceeds 10 mV and the mean frequency is 10 5/sec In other cells with somewhat lower resting potential the amplitude of the slow waves had about the same percentage relationship with the membrane potential as was found with the cell of Fig 1 On the most positive part of these waves irregular potentials were frequently seen After withdrawal of the microelectrode to a position just outside the cell hardly any potential fluctuations could be recorded at the low amplification used for the intracellular recording (record E) This shows that effects in B—D are trans membrane potentials At higher amplification of extracellular recording the slow waves were seen (record F)

It seems likely that the slow waves consist of rhythmically occurring synaptic potentials An attempt was made to analyze inhibitory and excitatory contributions by employing passage of current through the recording microelectrode The results were undecisive since a simultaneous extracellular recording was not made The expected action of a hyperpolarizing *IPSP* would be a phase reversal of the membrane potential changes relative to the extra cellular field potential

Discussion

The present intracellular recording from cortical cells reveals that corresponding to the slow wave activity recorded in the somatosensory areas after transection of the specific projection spinal pathways there are slow membrane potential changes in cortical cells. The relatively low membrane resting potential found in many cells is probably due to some damage to the cells caused by the microelectrode. This seems not to have interfered seriously with the function. The resting potential was usually stable and the rhythmical potential fluctuations continued throughout the recordings.

The amplitude of the potential changes may be as large as 30 % of the resting potential. It seems likely that the slow waves are caused by synaptic bombardment. This idea is also supported by the occurrence of small potential fluctuations on the positive part of the waves (Fig. 1 B—D). The source of this synaptic action is unknown but it may be a subcortical pacemaker. An attempt to disclose if inhibitory actions contribute was not successful but in further experiments the extracellular potential field will be used as reference. Long lasting inhibitory action has been found in cortical cells (Albe Fessard and Buser 1953, Albe Fessard 1960, Phillips 1959, Kandel, Spencer and Brinley 1961). It is likely that inhibitory actions contribute to the alpha rhythm as suggested by Andersen and Eccles (1962). If so, the potential variations in cells with normal membrane potentials probably are not as large as those described above since the magnitude of the inhibitory postsynaptic potential increases at low membrane potentials (Coombs, Eccles and Fatt 1955).

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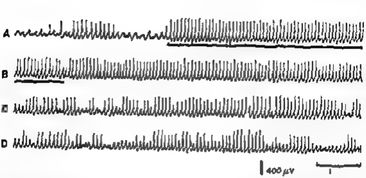

Fig 1 Continuous microelectrode recordings in the denervated forelimb area of SI in cat under light Nembutal anesthesia Effects of pressure on the contralateral hindlimb Horizontal line indicates the duration of the stimulation Note the increase in frequency amplitude and regularity of the slow waves Note also action potentials from single cells on the negative summit of the waves Negativity upwards

Results

I *Cortical effects obtained via central spinal pathways*

A *Effects on the slow wave activity in the denervated cortex* The spontaneous EEG activity in the parts of the somato sensory areas I and II corresponding to the projection from peripheral fields below the level of a spinal lesion interrupting the specific projection pathways is dominated by high voltage slow waves of a frequency of 8—12/sec (Andersson and Wolpow 1964) The amplitude of the waves is frequently rather constant but may show variations similar to the spindle activity in the surface recordings of the EEG Various stimuli applied below the spinal lesion changed the slow wave activity to greater regularity higher frequency and usually also larger amplitude (Fig 1) There was a rather long latency in the onset of these effects The changes in the activity outlasted the duration of the stimulation for a considerable time sometimes the resting activity did not return within a minute after the cessation of the stimulus

The changes in the slow wave activity could be induced by various types of peripheral stimuli The stimulus commonly used was pinching of the skin In some experiments muscles on the thigh were exposed and squeezing of the muscles was as effective in changing the wave activity as skin stimuli Forced movements of joints or radiating heat were also effective in changing the cortical activity On the other hand light stimuli such as brushing of the hair, jets of air or gentle movements of joints were usually ineffective

Stimuli of the above mentioned types were not equally effective in changing the slow wave activity when applied to different parts of the body below the level of the lesion It was usually found that stimulation in the part of the body corresponding to the projection area in which the microelectrode penetration was performed was most effective Thus in a preparation with transection of the specific projection pathways at C_3 the slow wave activity recorded

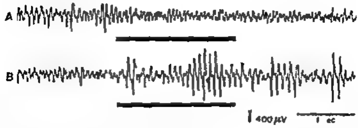

Fig. 2 Microelectrode recordings (inkwriter) in denervated hindlimb area in SI showing the effects of pinching the skin of the ipsilateral hindlimb at different depths of Nembutal anesthesia Record A shows the increase in frequency and decrease in amplitude induced in very light anesthesia *Shortly before taking record B 10 mg of Nembutal was given i v* The same stimulus now induces an increase in the amplitude of the waves Horizontal lines indicate duration of stimulation Negativity downwards

during a microelectrode penetration of the hindlimb area of SI or SII was most effectively changed from the hindlimbs In most experiments the best activation was obtained from the contralateral side but ipsilateral stimulation was also effective. In some experiments stimulation on the ipsilateral side was as effective as that on the contralateral side Stimulation of the forelimbs also induced changes in the slow waves but this activation was usually less pronounced and of shorter duration If the microelectrode penetration was performed in the forelimb area of SI or SII the best activation was obtained by stimulation of the contralateral and ipsilateral forelimbs but stimulation of the hindlimbs was also effective No strict topographical relation was found between the peripheral fields giving changed wave activity and the location of the microelectrode penetration

The cortical effects obtained via the ventral pathways were very sensitive to the depth of anesthesia It was necessary to allow at least 8 hours for the animal to recover from the ether anesthesia During this time successive doses of 5—10 mg Nembutal were administered i v to prevent spontaneous movements As the ether anesthesia gradually wore off and the animal became lightly anesthetized with Nembutal stimulation below the lesion started to be effective in changing the slow wave activity At a certain depth of anesthesia the stimulus gave an increase both in the frequency and the amplitude of the waves sometimes lasting for more than 1 min after the cessation of the stimulation (*Fig 1*) At this anesthetic level the frequency did not exceed 15 waves per sec during the stimulation These effects were usually found 3—4 hours after the administration of ether had been discontinued During this period the animal had been given a total amount of 15—20 mg kg of Nembutal If the anesthesia was allowed to become still lighter the increase in frequency was even more marked At this lighter anesthetic level there was frequently a decrease in the amplitude and simultaneously an increase in the frequency of the waves

(Fig 2 A) The upper limit for the wave frequency seems to be 17—18 waves per sec and is found only in very light anesthesia In a few instances of such light anesthesia the stimulation gave a longlasting desynchronization of the waves In these cases the initial effect of stimulation was an increase in the frequency of the waves and a gradual decrease in amplitude After a few seconds of stimulation the EEG was characterized by high frequency, low voltage activity The desynchronization outlasted the duration of the stimulus but the regular slow waves reappeared when the animal was left alone When 5—10 mg of Nembutal now was given to the animal the usual effect of stimulation was found i e increase in both amplitude and frequency After even further doses of Nembutal the change in the wave activity became more short lasting and was mainly an increase in the amplitude of the waves (Fig 2 B) The stimulation had usually no effect at all when an additional 10—15 mg dose of Nembutal had been given at the anesthetic level with maximum effects of stimulation on both amplitude and frequency of the waves mentioned above

B *Effects on single cells in the denervated cortex* As described previously (Andersson and Wolpow 1964) the cells in the denervated somato sensory cortex had a low rate of spontaneous activity Many of the cells fired on the most negative part of the waves other cells did not discharge in relation to the wave activity The effect of a strong stimulus applied below the lesion was usually excitatory with an increase in the frequency Units discharging spontaneously at irregular intervals frequently appeared on the summit of every wave (Fig 1) Simultaneously other cells not discharging in the unstimulated animal also started to fire The excitation of other cells during the stimulation was evident from differences in spike amplitudes The increased probability of firing on the waves was found both in connection with increased size of the waves due to the stimulation and when the wave amplitude increased spontaneously in a spindle like manner The activation of single cells outlasted the duration of the stimulus similarly as the increased wave amplitude (Fig 1) Cells spontaneously active without relation to the waves were usually also excited by the peripheral stimulus but most of these cells fired without relation to any particular part of the waves also during stimulation It was observed in many experiments that a change in anesthesia could change the pattern of the cell activity As described above the waves were usually decreased in size during light anesthesia in response to stimulation and increased in amplitude during stimulation in somewhat deeper anesthesia In some instances it was observed that a cell discharging without relation to the waves neither spontaneously nor during stimulation changed to firing synchronously with the waves after injection of a small amount of Nembutal

Some cells in the denervated cortex were inhibited by stimulation below the level of the lesion Also the inhibitory effect outlasted the duration of the stimulus Some of the cells which could be inhibited discharged spontaneously

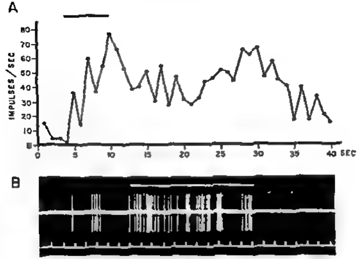

Fig 3 Microelectrode recording of a cell in the forelimb area of SI in a preparation with transection of the dorsal half of the spinal cord at the level of Th A graph of action potentials plotted against time in response to pinching of the contralateral hindlimb Note that the frequency has not yet returned to resting level 30 sec after cessation of the stimulus B oscillographic record of the discharge of the same unit showing the response to a jet of air to a small receptive field in the contralateral forefoot Negativity upwards
Horizontal lines indicate duration of stimulation

on the summit of the waves and a decrease in the wave amplitude or the abolishment of waves in response to stimulation decreased the activity in these cells

The receptive fields were very large for the cells activated in the denervated somato sensory cortex from below the level of the lesion Most of these cells were activated or inhibited from the entire body below the level of the lesion It must be emphasized that no units were found which were activated or inhibited from a small contralateral or ipsilateral area after interruption of the dorsal columns and the spino cervical tract However for many cells the strongest effect was obtained from the limb corresponding to the specific projection to that part of areas SI or SII where the cell activity was recorded Other cells were influenced as effectively from the ipsilateral as from the contralateral side and some cells showed the strongest activation when the ipsilateral side was stimulated

C *Effect on the EEG in the innervated cortex* There was a remarkable difference in the effect of a strong stimulation below the level of the spinal transection of the specific projection pathways on the EEG activity in the denervated and in the innervated cortex The same type of stimulation that gave rise to the slow wave activity in the denervated cortex as described above induced an increased frequency of low voltage activity (desynchronization in the surround

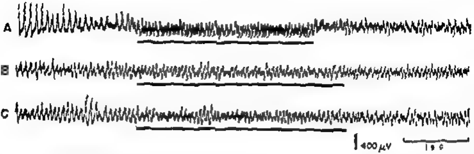

Fig 4 Microelectrode recording of the slow wave activity in the forelimb area of SII The dorsal half and the contralateral ventral quadrant of the spinal cord were transected at the level of Th A effects of electrical stimulation in descending direction of the dissected contralateral ventral quadrant (4V 10µsec 300/sec) B effects of pinching the skin of the ipsilateral hind limb C, effects of pinching the skin of the contralateral hindlimb

Horizontal lines indicate duration of stimulation Negativity upwards

ing innervated cortex This effect is quite similar to the EEG arousal reaction commonly observed in response to activating stimuli the desynchronizing effect is found in large cortical areas with suppression of the spindle activity for a considerable time Contrary to the local desynchronization found in the somatosensory cortex and elicited only from contralateral fields after a partial transection of the specific projection pathways described below the desynchronizing effect in the innervated cortex is obtained from both sides of the animal below the level of the lesion In some experiments in which the anesthesia was particularly light strong stimuli above the lesion could also give generalized suppression of the spindle activity in the innervated cortex

D *Effects on single cells in the innervated cortex* The effect on single cells in the innervated cortex of peripheral stimulation below the level of an interruption of the specific pathways has been studied most carefully in the forelimb areas of SI and SII In some experiments the effects have been studied in the auditory cortex and in the association cortex as well So far the visual cortex has not been investigated

The large majority of cells obtained in a microelectrode penetration in the somatosensory cortex in an animal with intact spinal pathways is characterized by specificity with regard to place and modality of the peripheral stimulus After transection of the specific projection pathways the effects of stimulation above the lesion were unchanged If for example the spinal lesion was made at Th_{10} the cells found in penetrations in parts of SI or SII corresponding to the forelimb were still activated from small contralateral skin fields (Fig 3B) or from circumscript subcutaneous areas In SI units activated by gentle movements of joints were also found However it was found that a very large number of these cells also could be activated from below the level of the lesion by strong stimuli of the same type as those activating cells in the denervated cortex Similarly as in the denervated cortex this effect could be elicited from

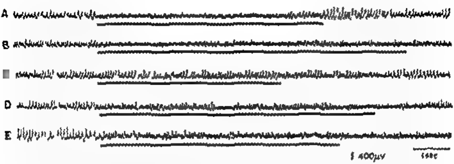

Fig 5 Microelectrode recordings in the hindlimb region of SI showing the effect of stimuli applied in different peripheral fields in a preparation with transection of the dorsal columns at C. See the text A the effect of light brushing on the contralateral hindpaw B the effect of pinching the contralateral hindpaw C, the effect of light brushing on the ipsilateral hind paw D the effect of pinching the contralateral forepaw
Horizontal lines indicate duration of stimulation Negativity upwards

large bilateral fields Further the effect outlasted the duration of the stimulus (Fig 3 A) Recordings from cells in the auditory cortex gave corresponding results Cells activated by sound or by click stimulation after a short latency could also be influenced by strong stimuli from below the spinal lesion Cells in the association cortex were usually not influenced by light stimuli but many cells were strongly affected by stimulation below the level of the lesion

E *Identification of a ventral spinal pathway mediating the cortical effects* Lundberg and Oscarsson (1962) have identified two ascending pathways in the ventral spinal cord both activated by stimuli eliciting the flexion reflex One of these pathways (labelled bVFRT by Lundberg and Oscarsson 1962) is a spinobulbar pathway activated from large bilateral receptive fields In addition this pathway receives a strong monosynaptic activation from a descending ventral pathway (Lundberg and Oscarsson 1962) In the present series of experiments one of the ventral pathways mediating the cortical effect in response to stimulation could be identified in the following type of experiments The dorsal columns and the dorsal part of the lateral funicles were transectioned at low thoracic level and the remaining ventral half of the cord was divided longitudinally One of the ventral quadrants was cut proximally and stimulated in descending direction with electrical pulses with a duration of 10μsec and a frequency of 100—300 per sec This stimulation activated very effectively the bVFRT ascending in the intact ventral quadrant (Lundberg and Oscarsson 1962) Electrical stimulation in descending direction of the dissected ventral quadrant gave an increased frequency of the low regular waves in the denervated cortex with a latency of less than 100 msec Fig 4 A This short latency indicates that the effects are due to activation of ascending spinal pathways It is unlikely that eventual co stimulation of autonomic fibres descending in the ventral quadrant would have caused the changed cortical activity by liberation of adreno-medullary hormones

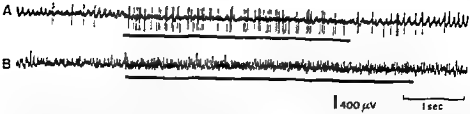

Fig 6 Microelectrode recordings in the hindlimb area of SI showing the effects of light continuous cutaneous stimulation in contralateral receptive fields in a preparation with transection of the dorsal columns at C See the text
Horizontal lines indicate duration of stimulation Negativity upwards

The effects obtained by electrical stimulation in descending direction were almost identical with those found by pinching in a bilateral receptive field below the level of the lesion (Fig 4B and C)

II *Cortical effects obtained after a partial lesion of the specific projection pathways*

In these experiments either the dorsal columns have been interrupted or the dorsal columns and most of the spino-cervical tracts have been transected but with an incomplete lesion of the contralateral spino cervical tract The ventral quadrants were intact in all these experiments The effects obtained in the partially denervated cortex in response to stimulation below the level of the lesion were more variable than those found after interruption of both the specific projection pathways

A *Transection of only the dorsal columns* Transection of the dorsal columns induces a slow wave activity in the hindlimb area of SI but no significant changes are found in the forelimb area of SI or in area SII (Andersson and Norrsell 1964 unpublished) Fig 5 illustrates the effects of different peripheral stimuli on the EEG in a penetration in the hindlimb area of SI The dorsal columns were cut at C_3 In this penetration no cells activated by light stimuli were found but an evoked potential of short latency was obtained in response to electrical stimulation of the contralateral hindpaw The spontaneous EEG showed a large wave activity with a frequency of 10 waves per second In Fig 5 record A the contralateral hindpaw was stimulated by light brushing There is a clear cut desynchronization of the large waves at the onset of stimulation but the wave activity returns shortly before the cessation of the stimulation In record C similar stimulation was applied to the ipsilateral hindpaw without any significant effect on the slow waves Pinching of the contralateral hindlimb (B) gives an immediate strong desynchronization but during the stimulation the slow wave activity returns The amplitude is decreased and the frequency increased The frequency and the amplitude did not return to the resting level within 30 sec A similar stimulus applied to the ipsilateral hindlimb (D) or the contralateral forelimb (E) gave no desynchronization but a longlasting decrease in amplitude and an increase in the frequency of the waves *In some*

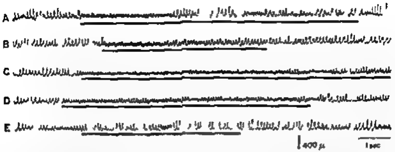

Fig 7 Microelectrode recordings from the forelimb area of SI in a preparation with transection of the dorsal columns and most of the contralateral spino-cervical tract at C_{3-4} showing the effect of various peripheral stimuli A light brushing on the contralateral forepaw B electrical stimulation of the contralateral forepaw (150 V 1 msec 95/sec) C pinching the skin of the contralateral forepaw D pressure on exposed muscles above the elbow in the contralateral forelimb E light brushing on the contralateral forepaw after complete transection of the contralateral spino-cervical tract

Horizontal lines indicate duration of stimulation Negativity upwards

penetrations light stimuli applied to the contralateral hindlimb gave activation of single cells and simultaneous desynchronization of the waves during the stimulation (Fig 6 A) In other penetrations in the hindlimb area of SI the spontaneous EEG was desynchronized In such penetrations light stimulation of the contralateral hindlimb induced a low voltage high frequency activity and cell activation (Fig 6B)

B *Transection of the dorsal columns and most of the spino cervical tract* Interruption of the dorsal columns and most of the spino-cervical tract causes the appearance of slow wave activity in both SI and SII in areas corresponding to the projection from the level below the lesion As indication that there still was a functional connection through the spino-cervical tract the evoked short latency potential has been used together with histological examination in serial sections of the parts of the spinal cord containing the lesions The transections of the dorsal columns and of the spino-cervical tract were made at slightly different levels The short latency potential evoked from below the lesion was usually reduced in amplitude by the lesion of the spino-cervical tract but the latency was unchanged The effect of peripheral stimulation of the EEG activity is illustrated in Fig 7 The microelectrode penetration was made in the forelimb area of SI No cells activated by light stimuli to the contralateral forelimb were found in this penetration However light brushing of the contralateral forepaw gave an initial desynchronization of the wave activity but the slow waves returned during the stimulation (Fig 7 A) Electrical stimulation of the skin in the same paw had a similar effect (B) Strong stimulation (pinching) in the same area gave an initial desynchronization but during the stimulation the waves returned with an increased frequency (C) Pressure on muscles in the contralateral forelimb gave increased frequency of the waves without any initial

desynchronization (D) An increase in the frequency was obtained also by pinching the ipsilateral forelimb and the hindlimbs (not illustrated) The contralateral spino cervical tract was then transected completely The effect of brushing on the contralateral forepaw after this lesion is shown in Fig 7E The wave activity is unchanged during the stimulation Pinching the contralateral forepaw gave increased frequency of the waves without any initial desynchronization The effects of strong stimuli in other areas were unchanged compared with the effects before the complete transection of the spino cervical tract

Discussion

A transection of the dorsal part of the spinal cord interrupting the specific projection pathways abolishes completely the short latency cortical potentials evoked by adequate or electrical peripheral stimulation of the skin of a cat anesthetized with Nembutal (Norrsell and Voorhoeve 1962, Andersson 1962a) The strong generalized cortical effects found in this investigation must thus be mediated via pathways located in the remaining intact ventral quadrants It has been possible to identify one ventral spinal pathway mediating the cortical effects namely the bilateral ventral flexor reflex tract (bVFRT) of Lundberg and Oscarsson (1962) Neurones of this tract are polysynaptically activated from bilateral fields by the flexor reflex afferents (FRA) group II and group III muscle afferents cutaneous and high threshold joint afferents The pathway terminates in the lower brain stem in the region of the lateral reticular formation The type of stimuli giving the above mentioned cortical effects would give strong activation of the bVFRT pinching or heating of the skin pressure or massage of muscles and forced joint movements The transmission to bVFRT is under a strong inhibitory control of a descending pathway located in the dorsal part of the lateral funicle This pathway will be transected simultaneously with interruption of the spino cervical tracts (Holmqvist Lundberg and Oscarsson 1960) Stimulation of the FRA below the level of the spinal lesion will consequently give a much more effective activation of the bVFRT than stimulation in peripheral fields above the lesion where the inhibitory control is intact The abolished inhibition of the transmission from the FRA to ascending pathways is in all likelihood the reason for the difference in cortical effect obtained by similar stimuli above and below the level of the lesion

Stimulation below the level of a spinal lesion interrupting the specific projection pathways results in a generalized change in the cortical activity but the effect in the denervated cortex is different from that found in the innervated cortex At a suitable depth of anesthesia a strong stimulus gives a longlasting increase in amplitude and frequency of the regular slow wave activity in the denervated somatosensory areas but a desynchronization with abolished spindle activity and increased low voltage and high frequency activity of

similar duration in other parts of the cortex In the unanesthetized preparation generalized cortical desynchronization is easily obtained in response also to light stimuli and high frequency electrical stimulation of afferent nerves (Pompeiano and Swett 1962b) but in the Nembutalized animal used in the present investigation no generalized cortical effect could be elicited in response to light stimuli either above or below the level of the lesion The anesthesia increases the threshold for arousal and it is necessary to use massive activation of the FRA in order to obtain the cortical arousal reaction It is not likely that the absence of generalized arousal in response to light stimuli below the level of the lesion is due to the interruption of the specific projection pathways since for the following reasons it is unlikely that these pathways play any important role in these generalized effects 1) There is no anatomical evidence that fibres ascending in the medial lemniscus terminate or have collateral connections to the reticular formation (Matzke 1951 Rossi and Brodal 1957 Bowsher 1958) 2) The effects mediated via the dorsal columns in a preparation with all other pathways interrupted are not generalized but independent of the intensity of stimulation are confined to those parts of the somato-sensory cortex which correspond to the stimulated peripheral area (Andersson unpublished)

There are good reasons to assume that the effects mediated via ventral pathways and giving generalized cortical activation with desynchronization in the innervated cortex and wave activation in the denervated cortex are mediated via the same afferent system 1) Both types of cortical effects are elicited by the same type of stimulation below the level of the spinal lesion 2) The activation of waves in the denervated cortex and the desynchronization in the innervated cortex have a similar duration and outlast the duration of the stimulus 3) Both effects have a similar sensitivity to anesthesia 4) In some instances with the animal very lightly anesthetized stimulation can induce a desynchronization also in the denervated cortex

The cortical effects mediated via the ventral spinal cord are in all likelihood identical with the arousal phenomenon originally described by Moruzzi and Magoun (1949) These authors found a generalized desynchronization in response to electrical stimulation of the reticular formation The cortical arousal reaction obtained from this area is mediated via the ascending activating system (for references see Jasper 1960) It has been assumed that this system relays in the intralaminar thalamic nuclei and a generalized cortical activation can be obtained also from these nuclei by electrical stimulation at high frequency Although the activating effects elicited via the ascending activating system are generalized there are some trends of topographical organization (Jasper Naquet and King 1956) An indication of a topographical arrangement was also found in the effects mediated via the ventral pathways in the present experiments Thus stimulation of the contralateral limb corresponding to the location of the recorded slow wave activity usually gave the most ef

fective activation of the slow waves although the general rule was that the differences in effect between different stimulated fields below a complete lesion of the specific projection pathways were only quantitative

Electrical stimulation with low frequency within the ascending activating system can also give synchronized cortical activity i e the recruiting response *This synchronization is a generalized cortical phenomenon* This type of activity is obtained in response to electrical stimulation in thalamic nuclei (for references see Spencer and Brookhart 1961) It has later been found that similar cortical activity can be obtained by stimulation in the lower brain stem (Magnes, Moruzzi and Pompeiano 1961 Favale *et al* 1961) and by stimulation of peripheral nerves (Pompeiano and Swett 1962a 1962b) It has not been possible however to find any nervous structure from which only a cortical synchronizing effect can be elicited Increase of the frequency of the stimulation always changes the response into desynchronization (arousal) It is interesting that the maximum frequency of the waves in the denervated cortex during peripheral stimulation is in the same range as the maximum frequency of synchronized wave activity that can be obtained with electrical stimulation of peripheral nerves or of the ascending reticular system This indicates that the low frequency electrical stimulation gives neuronal activity of a temporal pattern that can enhance the pacemaker mechanism responsible for slow waves When the stimulation rate exceeds a critical level the temporal pattern of the neuronal activity will be changed and the pacemaker mechanism cannot exert its synchronizing action Adequate peripheral stimulation will act the same way as high frequency electrical stimulation The wave activation obtained in the denervated cortex suggests either that the effect of the ascending activating system upon the pacemaker mechanism has been changed by the transection of the specific projection system or that due to the cortical state the pacemaker activation has a different effect on the cortex The lesion in the spinal cord does *not in itself prevent the generalized desynchronizing effects* elicited by peripheral nerve stimulation (Pompeiano and Swett 1962a)

The importance of the impulses in the specific projection system for the desynchronization in its cortical projection area is clearly illustrated in the experiments with partial transection of the specific projection pathways It was regularly found that the slow wave activity was more or less desynchronized in areas of SI and SII by light stimuli applied to the corresponding *contralateral* peripheral field This effect was similar in duration to the stimulation and the slow waves appeared immediately when the stimulation ceased Strong *stimuli* applied to the same field gave a desynchronizing effect initially but during prolonged stimulation the regular slow waves returned gradually and showed an increased frequency Stimuli to other peripheral fields gave no *desynchronizing* effect Instead the slow waves increased in frequency and *commonly also* in amplitude similar to preparations with complete transection of the *specific* projection system In preparations with cut dorsal columns and only a small

fraction of the contralateral spino cervical tract intact the initial desynchronizing and the following slow wave activating effect was very striking The desynchronizing effect is probably due to quickly adapting fibres After transection of the remaining part of the specific projection pathways no desynchronizing effect could be elicited

It has been generally supposed that cortical desynchronization is due to the tonic activity in the ascending activating system (Pompeiano and Swett 1962a) The present findings indicate that the specific projection system is also important in preventing slow wave activity not by collateral effects on the activating system but via a different mechanism It is evident that impulses in the specific projection system are capable of preventing the localized slow wave activity There are at least two possibilities for such an effect 1) Inhibition of a pacemaker mechanism at thalamic level supposedly responsible for the slow waves in the denervated cortex Synchronizing as well as desynchronizing cortical effects can be elicited from the intralaminar thalamic nuclei These effects are generalized although there is a tendency to topographical organization An inhibitory effect on a pacemaker in the intralaminar nuclei as the explanation for the absence of slow wave activity in the innervated cortex would probably require inhibition of cells in these nuclei by impulses in the specific projection pathways Although such inhibition has been reported it does not seem to be common (Albe Fessard and Kruger 1962) It should also be noted that the slow waves in the denervated cortex appear to be very localized not only with regard to the forelimb and hindlimb areas but also to regions within the hindlimb area of SI after transection of the dorsal columns Such a localized release cannot be explained on the basis of our present knowledge of the organization of the ascending activating system 2) The slow wave activity is prevented in the innervated cortex by an effect of the specific projection system on neuronal elements at a cortical level Such an explanation would fit with the strict localization of the appearance of slow waves The specific projection system is topographically organized in its cortical projection and consequently transection of the pathways would abolish the transmission to the cortical area corresponding to the projection from below the lesion This hypothesis can al o explain why stimulation of the specific projection system gives a desynchronization in the corresponding cortical area after a partial lesion

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Vasoconstrictor Action of Acetylcholine on Kidney Blood Vessels

By

A Åström J Crafoord and U Samelius Broberg

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Abstract

Åström A J Crafoord and U Samelius Broberg *Vasoconstrictor action of acetylcholine on kidney blood vessels* Acta physiol scand 1964 *61* 159—164 Changes in the renal vascular resistance of cats and dogs have been studied employing a constant flow perfusion technique Close intraarterial injections of acetylcholine in large doses usually greatly increase resistance This effect is enhanced by neostigmine and abolished by atropine It is not abolished by ganglionic blockade adrenolytic agents or pretreatment of the animals with reserpine The effect has been observed in kidneys with intact nerve supply as well as after denervation. When the action of acetylcholine is tested during an infusion of lidocaine into the renal artery the response is slightly reduced The mechanism of action is discussed and it is concluded that in this preparation the vasoconstrictor action of acetylcholine is due to a direct action on the vascular smooth muscle

Although acetylcholine ACh as a rule dilates peripheral vessels a vasoconstrictor action has also been observed in several vascular regions e g the pulmonary and coeliac venous beds (for references see Furchgott 1955) the placental vessels (Ueda 1931 Euler 1938) and the vessels of the isolated rabbit ear (Burn and Robinson 1951 Kottegoda 1953)

During a study of the influence of vasoactive agents on the kidney vessels of the cat it was found that in large doses ACh often produced a vasoconstriction In the present investigation this action has been analyzed with particular attention directed towards the possibility that the constrictor action of ACh might result from an indirect action e g through the release of catecholamines (Kottegoda 1953 Burn and Rand 1959)

Methods

Cats were anesthetized with intraperitoneal sodium pentobarbital 35 mg/kg The left kidney was approached through a midline abdominal incision and the renal artery cannulated near the aorta with a polyethylene catheter The other end of this catheter was inserted into the right femoral artery A pump was included in the arterial loop thus established Intravenous injections were made into an external jugular vein close intraarterial injections were given in the arterial loop on the inflow side of the pump The volume of each arterial injection was 0 2 ml In some of the experiments nerve fibers running along the renal artery were prepared for electrical stimulation Square wave pulses with a voltage of 5—10 V, a frequency of 2—8 pulses/sec and a duration of 1—2 msec were used

Resistance changes in the kidney blood vessels were measured as pressure variations in the arterial loop between the pump and the kidney Systemic arterial pressure was recorded from the left femoral artery All pressures were measured with strain gauge manometers and recorded on a polygraph (model 5 Grass Instrument Company Quincy Mass USA)

Blood flow through the kidney was set with the pump to give an arterial pressure of about 110 mm Hg and varied between 2 and 4 ml/g/min Small flow adjustments were occasionally necessary to maintain this perfusion pressure during an experiment A slow infusion of isotonic glucose with 3—5 % dextran was usually given to keep the animal in good condition

In one group of experiments the kidney was denervated as follows An arterial loop was established as described above A parallel venous loop connecting the left renal vein with the right femoral vein was also made and the left ureter was cannulated The kidney was then dissected free from its surroundings and placed in a thermoregulated bath containing Ringer s solution

The following drugs were used angiotensin (Hypertensin® Ciba) atropine (sulfate) BOL (2 brom lysergic acid diethylamide) dextran (Rheomacrodex® $\overline{Mw}$ 40 000 Pharmacia) DHE dihydroergotamine (methanesulfonate Dihydergot® Sandoz) DMPP (1 1 dimethyl 4 phenyl piperazine iodide) hexamethonium (-chloride Dylensin® Leo) lidocaine (hydrochloride Xylocain® Astra) neostigmine (methylsulfate Neostigmin® Leo) nicotine (hydrogen tartrate) pentobarbital sodium (Nembutal® Abbot) phentolamine (methane sulfonate Regitine® Ciba) reserpine (Serpasil® Ciba) Doses are given as salts We thank the manufacturers for kindly supplying these drugs

Results

All results given below were reproduced in at least 3 expts unless otherwise stated

The effect of ACh depended on the dose given Small doses (0 002—0 02 μg) caused a decrease in perfusion pressure medium doses (0 02—0 2 μg) often produced a biphasic response — a brief increase followed by a decrease — while large doses (0 2—200 μg) produced an increase in perfusion pressure In 5 expts out of 40 ACh caused only a fall in perfusion pressure regardless of the dose It was often observed that the first pressor response was evoked with a very small dose of ACh while later a larger dose was needed to obtain the same response The response to this larger dose would then remain con

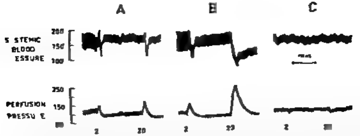

Fig 1 Effects of close intraarterial injections of acetylcholine ( ) in μg as indicated A control B after neostigmine (30 μg/kg i.v) and C after atropine (0,2 mg/kg i.v) Pressures in mm Hg

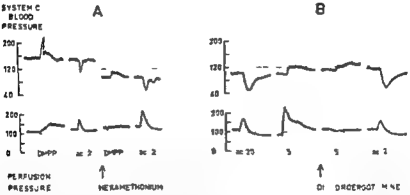

Fig 2 A. Denervated kidney Effect of DMPP 200 μg (DMPP) and acetylcholine 0.2 μg (ac .2) before and after the administration of hexamethonium 10 mg/kg i v B Kidney with intact nerves Effect of acetylcholine 20 μg (ac 20) and adrenaline 5 μg (a 5) before and after the administration of DHE, 0 5 mg/kg i v Pressures in mm Hg

stant for several hours provided that the interval between consecutive injections was at least 2 min

The effect of ACh was tested in animals that had been pretreated in various ways

The pressor action of ACh was potentiated by neostigmine in a dose of 30 mg/kg i v and abolished by atropine in a dose of 0 2 mg/kg i v Fig 1)

Hexamethonium was given i v in a dose of 10—20 mg kg The pressor action of ACh was not changed but hexamethonium blocked the effect of the ganglionic stimulating agents DMPP and nicotine Fig 2 A)

Dihydroergotamine was given i v in a dose of 0 5 mg kg The pressor action of ACh was the same before and after this treatment which blocked the pressor action of adrenaline (Fig 2 B) noradrenaline and electrical stimulation of the renal nerve Similar results were obtained with phentolamine 2 expts) The pressor action of ACh was not affected by BOL

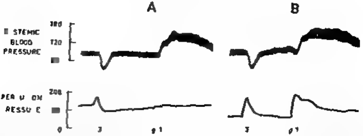

Fig. 3. A. Effect of acetylcholine 3 µg (ac 3) and angiotensin 1 µg (ang 1) tested while lidocaine (1.5 mg/kg/min) is added to the blood perfusing the kidney. B. The same tests after the lidocaine addition has been discontinued.

Reserpine was given i.p. in a dose of 5 mg/kg 24 hours before the experiment. The renal vascular bed in these animals did not contract in response to stimulation of the renal nerve but the pressor response to ACh was obtained in 6 expts out of 8.

The pressor action of ACh was tested during experiments in which a 10 % lidocaine solution was slowly infused into the blood of the arterial loop so the cat received 1—2 mg/kg/min. The response was reduced though not abolished and reappeared when the lidocaine infusion was discontinued. The vasoconstriction produced by angiotensin was diminished to a similar degree (Fig. 3).

Experiments with denervated kidneys gave basically the same results as those in which the nerve supply of the kidney was intact. In one expt. of 7 no pressor action was produced by ACh.

Vasopressor action of ACh was also obtained in the renal blood vessels of dogs (3 expts.) but not of rabbits (3 expts.).

Discussion

The increase in resistance caused by large doses of ACh given by close i.a. injection is most likely due to an active vasoconstriction, although the data here do not indicate which particular part of the renal vascular bed is primarily involved. The reason why ACh produced only vasodilatation in 5 expts of 40 has not been established.

The technique used seems to rule out any primarily extrarenal action of ACh. The effects observed were not changed by denervation and thus independent of extrarenal nerves. Vasoconstriction was observed in the renal vascular bed before the ACh had reached the systemic circulation and the usual drop in arterial pressure had occurred (Fig. 2). These observations seem to rule out the possibility that the effect is either part of a general vasoconstrictor reflex activity or mediated by an extrarenal release of catecholamines. This conclusion is further supported by the results with reserpine and DHF

Reserpine pretreatment renders electrical stimulation of the renal nerve ineffective and DHE abolished the vasoconstriction of catecholamines but neither drug changed the action of ACh. These observations also seem to rule out the possibility of a peripheral release of ACh of catecholamines in the manner suggested by Burn and Rand (1959).

In the kidney ACh could possibly act indirectly via local nervous structures. The kidney is known to contain autonomic ganglia which can be stimulated by nicotine or DMPP to produce an increase in renal vascular resistance as shown by Page and McCubbin (1953). These authors also showed that the vasoconstriction elicited by nicotine or DMPP could be prevented by a ganglionic blocking agent but not by atropine. In our experiments the vasoconstrictor action of ACh was not abolished by hexamethonium in a dose sufficient to block the action of nicotine or DMPP and an action of ACh on these ganglia may therefore be excluded.

The results with a local anesthetic (lidocaine) also indicate that ACh acts independently of nervous structures. A decrease in the constrictor effect of ACh was observed only after high doses of lidocaine which also decreased the effect of angiotensin. In high doses local anesthetics have been found to be nonspecific inhibitors of several agents producing constriction of isolated strips of the rabbit aorta (Åström 1964).

The evidence thus indicates that the vasoconstrictor effect of ACh is a direct action on the smooth vascular muscle. The observation that neostigmine enhances and atropine blocks this action supports this conclusion. The situation would then be best compared with that observed in several isolated segments and strips from different arteries and veins. The contraction produced in such preparations by ACh has also been found to be inhibited by atropine while hexamethonium and DHE had no effect (Furchgott 1955).

The results with ACh on kidney vessels may be compared with those obtained on the umbilical vessels. Working with this preparation which has no nerve supply Ueda (1931) observed a dose response relationship for ACh similar to that observed in this study. Euler (1938) and Eliasson and Åström (1955) reported that ACh sometimes caused the vessels of the human placenta to constrict and that this effect could be blocked by atropine. Euler also occasionally observed week dilatator effects of ACh.

It is worth noting that unlike the placenta the kidney has a nervous supply with adrenergic and according to recent histochemical studies also cholinergic fibers the terminals of which are distributed almost exclusively along the arteries and arterioles of the renal cortex (Coupland 1962). The adrenergic fibers are known to be vasoconstrictor fibers but the function of the cholinergic fibers is unknown.

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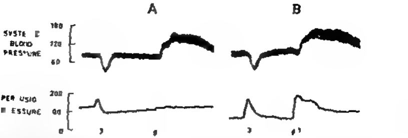

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The technique used seems to rule out any primarily extrarenal action of ACh The effects observed were not changed by denervation and thus independent of extrarenal nerves Vasoconstriction was observed in the renal vascular bed before the ACh had reached the systemic circulation and the usual drop in arterial pressure had occurred (Fig 2) These observations seem to rule out the possibility that the effect is either part of a general vasoconstrictor reflex activity or mediated by an extrarenal release of catecholamines This conclusion is further supported by the results with reserpine and DHE

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The Interrelationship of Some Factors Influencing Renal Blood Flow Autoregulation

By

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Abstract

Folkow B and J Langston *The interrelationship of some factors influencing renal blood flow autoregulation* Acta physiol scand 1964 *61* 165—176 — A study has been performed on cats concerning the interrelationship of active and passive local mechanisms for producing renal blood flow autoregulation as counteracted by the centrally directed influence of the renal vasoconstrictor fibres The experiments indicate that the active mechanism dominates completely at moderate or higher blood pressure levels but also that the passive one contributes significantly at lower pressures or whenever the active mechanism fails However these autoregulatory mechanisms can be more or less completely overwhelmed by a vasoconstrictor fibre activity which makes the renal vascular circuit behave as a passive elastic tube system Some implications of these findings are briefly discussed

The mechanisms responsible for autoregulation of regional blood flow have aroused considerable interest to the extent that a recent international symposium (1963) was devoted to this problem Especially the pronounced autoregulation of renal blood flow has been a matter of much controversy and the debate has mainly been concentrated to the question whether under physiological circumstances an active local vascular adjustment or a purely passive mechanism is responsible for the autoregulation

Concerning the first mentioned possibility the hypothesis of Bayliss (1902) implying that the distension offered by the blood pressure might serve as an adequate stimulus for the vascular smooth muscles has been much discussed Such a concept which seems to be in good agreement with by now well known characteristics of other types of myogenically active smooth muscles has in its essential elements been experimentally validated at least in some vascular beds

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Changes in transmural pressure have thus been found to affect vascular tone in several major circuits of the systemic circulation, this effect being essentially confined to the precapillary resistance vessels (c f Folkow 1962) Similar mechanisms seem to be operating in the kidney (e g Miles, Ventom and de Wardener 1954 Waugh 1958 Thurau and Kramer 1959), which similarly to the situation in other vascular circuits seem to be largely confined to the pre glomerular vessels (Thurau and Wober 1962) Local nervous networks and metabolic factors appear to be excluded as responsible for renal blood flow autoregulation

However, it is not entirely impossible that in such a highly specialized tissue as the kidney, where blood flow is intimately coupled to renal regulation of the organism s fluid electrolyte equilibrium the intrinsic control of the blood flow is in some way directly linked to the fluid electrolyte exchange rather than to the transmural blood pressure For instance at the abovementioned symposium Guyton (1963) called attention to the peculiarly close proximity between the macula densa and the afferent arteriole and pointed out that one must be open to the possibility that this might imply some type of a vasoregulatory design As however such a hypothetical mechanism like the transmural pressure, may be considered to influence in the final end the preglomerular vascular smooth muscles the term active mechanism is here used in its broadest sense to include any type of intrarenal mechanism affecting preglomerular vascular resistance independent of the stimulus type

On the other hand, it has convincingly been shown that an entirely passive mechanism also can produce autoregulation of renal blood flow, though generally it does not seem to be very powerful This mechanism is a consequence of the enclosure of the kidney within a rigid capsule which tends to curtail the change of effective pressure head produced within the kidney when arterial pressure is raised (c f Hinshaw *et al* 1959) This phenomenon is easily illus trated even in model experiments but it is obvious that in case effectively operating active preglomerular adjustments do take place they will minimize changes in intrarenal pressure along with arterial pressure shifts and hence put the passive mechanism more or less out of operation The question is therefore which of the two mechanisms normally dominates renal hemody namics or under which circumstances the one or the other might dominate

The problem of renal blood flow autoregulation is made still more complex by the observation that the phenomenon can be entirely absent under cir cumstances that hardly can be called unphysiological and where no experi mental artifacts seem to be involved Thus de Wardener and Miles (1952) observed that upon hemorrhage which caused renal vasoconstriction flow autoregulation was impaired or lost Similarly one of the present authors has reported a consistent absence of flow autoregulation in the kidneys of dogs where the low blood flow figures suggested the presence of renal vasoconstriction (Langston *et al* 1959)

Thus at first sight quite contradictory results have been obtained in different laboratories The question then arises whether these contradictions are only apparent insofar as the results merely reveal the existence of several, in nature different but *per se* truly physiological mechanisms The balance between them may then depending on the actual circumstances be so shifted that the integrated response can differ considerably It might therefore still be justified to add to the by now imposing series of studies dealing with renal autoregulation in case the abovementioned apparently contradictory results could be reproduced in one and the same series of animals, and analyzed as to the interrelationship of the mechanisms involved The present experiments which were briefly discussed at the recent symposium on blood flow autoregulation (Folkow 1963) are an attempt along these lines

Methods

Results from 24 cats weighing between 1 5 and 2 5 kg are represented in this study Ether was administered as a preliminary anesthetic after which 40 to 50 mg/kg of chloralose was given as an i v infusion Normal body temperature was maintained with the aid of a heating pad and a heat lamp A tracheal cannula was inserted and the two common carotid arteries and vagal nerves were freed so that the carotid arteries could be clamped and the vagal nerves cut in the course of the actual experiment The arterial pressure was recorded from one of the femoral arteries by means of a mercury manometer and a kymograph

The abdomen was opened by a midline incision and the omentum spleen colon and greater part of the small intestine were extirpated The right adrenal gland was excluded from the circulation by ligatures placed around its vessels The left adrenal gland was in most experiments denervated by ligation of its nervous supply as it enters the border of the gland great care being taken not to damage the vasomotor nerves to the kidneys In the course of the experiment the renal vasomotor nerves could be blocked by infiltration of the renal pedicle with xylocain.

An adjustable clamp was placed around the aorta between the diaphragm and the renal arteries so that the arterial pressure perfusing the kidneys could be varied in a stepwise fashion The animal was heparinized and the blood flow from the left kidney was recorded This was accomplished by cannulating the renal vein taking care not to damage any nerves in this area The renal venous outflow was then diverted through a low resistance siliconized optical drop recorder unit operating an ordinate writer The venous outflow was returned to the animal through the external jugular vein thus forming a closed external system In several of the experiments the blood flow from the right kidney was simultaneously recorded in the same manner

The renal venous pressure was measured with a water manometer attached to the inflow catheter of the drop recorder and was found to be between 5 and 10 mm Hg and remained approximately the same throughout each experiment The renal perfusion pressure referred to throughout this report was calculated as the difference between the mean arterial pressure and the renal venous pressure

After cutting both vagal nerves the two common carotid arteries were clamped while the renal perfusion pressure was maintained constant during the reflex blood pressure rise by increasing the resistance at the aortic clamp The reflex increase of sympathetic discharge induced by the carotid occlusion was found to have no or only negligible effect on renal blood flow resistance as has previously been shown to be the

case as long as no additional excitatory influences affect the sympathetic discharge to the kidneys (c f Löfving 1961 Folkow Johansson and Löfving 1961) The aortic clamp was then fully opened so that the renal vessels were perfused at a pressure head of 200—250 mm Hg The renal perfusion pressure was then decreased in approximately 20 mm Hg steps by increasing the resistance at the aortic clamp and the renal blood flow was allowed to stabilize at each pressure level This procedure was repeated and varied in several ways increasing and decreasing the perfusion pressure over a range from 40—60 mm Hg to 200—250 mm Hg Pressure flow curves were then deduced from these recordings

The effect of i v infusion of noradrenaline on the renal pressure flow relationship was then studied in 6 of the cats in the following manner Noradrenaline was given at a constant rate so adjusted as to maintain renal blood flow resistance at a steady level about 2—3 times higher than before the infusion the resistance measurements being performed at a perfusion pressure of 100 mm Hg This usually required from 2 to 5 μg of noradrenaline per kg body weight per min The renal pressure flow relationships were again determined and compared with those obtained before and after the period of noradrenaline infusion Great care was taken to ensure that the increased renal resistance was really kept essentially the same by checking repeatedly the flow resistance at 100 mm Hg perfusion pressure

In most of the other experiments the effect of increased renal vasoconstrictor fibre activity upon blood flow autoregulation was studied in the following way The easiest way to induce a powerful and fairly stable neurogenic vasoconstriction within the kidney proved to be the Cushing reflex To accomplish this a metal catheter connected to a pressure bottle was introduced into the cranial cavity by a small hole drilled in the parietal bone Increasing the pressure in the bottle to nearly that of the arterial blood pressure caused a prompt rise in blood pressure accompanied by a powerful and often fairly constant renal vasoconstriction when intracranial pressure was maintained at a high level Before initiating the Cushing reflex artificial respiration was started at a rate and tidal volume which barely suppressed the spontaneous respiration

When the neurogenic restriction of the renal blood flow had stabilized a pressure flow relationship was determined and compared with the pressure flow relationships obtained before and after the Cushing reflex In some of the experiments where blood flow was simultaneously recorded from both kidneys the left one was denervated so that the pressure flow relationship of one denervated and one normally innervated renal vascular bed could be directly compared before during and after the induction of a Cushing reflex

In three experiments in which both renal blood flows were recorded simultaneously the left kidney had been cautiously decapsulated to eliminate the possible influence of the rigid enclosure upon renal pressure flow relationships In these three experiments the renal vasoconstrictor nerves were left intact and it was checked as earlier described that carotid occlusion *per se* did not produce any significant neurogenic vasoconstriction The renal autoregulatory ability was then studied simultaneously in the decapsulated and the intact kidneys before during and after a period of intense renal vasoconstriction produced by the Cushing reflex

In five additional experiments, where blood flow autoregulation was to be studied in decapsulated kidneys the kidney was enclosed in a small plethysmograph in order to record simultaneously such changes in renal volume which might take place in connection with changes in arterial pressure The urether was dissected free opened distally and allowed to empty the slow urine flow outside the plethysmograph along the vascular hilus It was assumed in these experiments that in case active autoregulatory events took place and were essentially concentrated to the precapillary resistance vessels the

changes in arterial pressure would only to a negligible extent be transmitted to the more distensible postcapillary section of the renal vascular bed and therefore only minimal volume changes would occur. If, on the other hand, no precapillary resistance adjustments would take place at, say, a 50 mm Hg arterial pressure rise and if it is assumed that about 4/5 of the total renal flow resistance is proximal to the tubular capillary level, a mean rise in pressure of some 10 mm Hg would occur at the tubular capillary end of the renal venous compartment, resulting in a substantial distension of the veins in case tissue pressure in the decapsulated kidney does not increase proportionally. An increase in organ volume would then be expected to occur and its extent could be directly compared with the volume increase obtained when renal venous pressure was raised in small steps under the control of the venous pressure manometer by a partial obstruction of the venous outflow tube. If the volume of the decapsulated kidney regularly increased significantly at e.g. a venous pressure rise of 5 mm Hg, proving that in this situation a prompt tissue pressure rise does not curtail the intravascular pressure rise, while at the same time the volume increased far less or not at all when arterial pressure rose, say, 50 mm Hg, such a finding would strongly suggest that the autoregulation of the blood flow is due to resistance adjustments proximal to the tubular capillaries and not due to a tissue pressure rise only. It was considered to be necessary to check such a possibility as it could not be taken for granted that the rigid renal capsule is alone responsible of raising tissue pressure when arterial pressure is raised.

Results

The influence of noradrenaline infusion on renal blood flow autoregulation

Figure 1 represents results obtained from 6 expts. in which the autoregulatory ability of the kidney was determined before, during and after noradrenaline infusion. Fig. 1A represents results obtained before the drug infusion and indicates that the mechanisms responsible for autoregulation were performing fairly efficiently. As the arterial pressure perfusing the kidneys was increased from 100 to 180 mm Hg, there was a concomitant mean increase in renal resistance of about 32 per cent.

The autoregulation is largely abolished during the renal vasoconstriction caused by the noradrenaline infusion, as indicated by Fig. 1B. When now the renal arterial pressure was increased from 100 to 180 mm Hg, renal flow resistance decreased some 8 per cent. It was also in general found that the autoregulatory ability was more effectively abolished the more the renal vessels were constricted.

Fig. 1C represents the results obtained 15 min after the noradrenaline infusion was stopped, showing that the mechanisms responsible for renal autoregulation were again capable of increasing the vascular resistance to about the same extent as before the noradrenaline infusion when arterial pressure was increased.

The influence of renal vasoconstrictor fibre activity on renal blood flow autoregulation

The effect of the increased activity of the vasomotor centre, as elicited by the Cushing reflex, on the autoregulatory ability of the kidney was studied in 10

case as long as no additional excitatory influences affect the sympathetic discharge to the kidneys (c f Lofving 1961 Folkow, Johansson and Lofving 1961) The aortic clamp was then fully opened so that the renal vessels were perfused at a pressure head of 200—250 mm Hg The renal perfusion pressure was then decreased in approximately 20 mm Hg steps by increasing the resistance at the aortic clamp and the renal blood flow was allowed to stabilize at each pressure level This procedure was repeated and varied in several ways increasing and decreasing the perfusion pressure over a range from 40—60 mm Hg to 200—250 mm Hg Pressure flow curves were then deduced from these recordings

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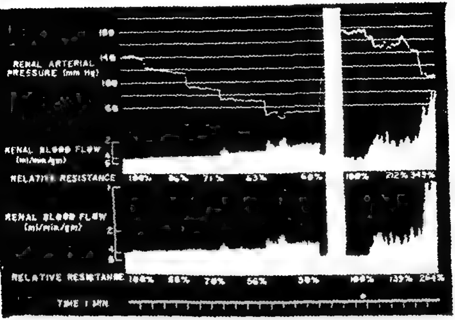

Fig 3 Effect of renal de apsulation on the renal pressure flow relationship The blood fl w from the decapsulated l ft kidney is shown by the upper flow recording and the blood flow from the right kidney with capsule intact is shown by the lower flow recording The first portion of this figure represents results obtained before the act ation of the Cushing reflex The second section epresents results obtained during renal vasoconstriction produced by the Cushing reflex The resistance is gi en at each pressure level for each kidney as the per cent of the re istance at the first pressure level

When now arterial pressure was increased from 100 to 180 mm Hg renal blood flow resistance decreased about 20 per cent In these experiments also it was observed that the autoregulatory ability was decreased the most in those kidneys where the most powerful neurogenic vasoconstriction had been in duced Fig 2C represents the situation in ix of the ten animals when the Cushing reflex manoeuvre was repeated after the renal vasoconstrictor fibres had been blocked by xylocain In 3 of these animals the blood flow was recorded from the left kidney only which was denervated after the results shown in Figure 2B were obtained In the remaining 3 expts the blood flow from both kidneys were simultaneously recorded where only the left kidney was denerva ted leaving the nervous supply to the right kidney intact The results from the right innervated kidneys of these 3 animals are therefore represented in Fig 2A and 2B while the results from the denervated left kidneys are represented in Fig 2C These results show that the renal autoregulatory ability is over powered by the sympathetic discharge induced from the central nervous system only as long as the renal vasocons rictor fibres remain intact It should here be pointed out that the adrenal glands were denervated

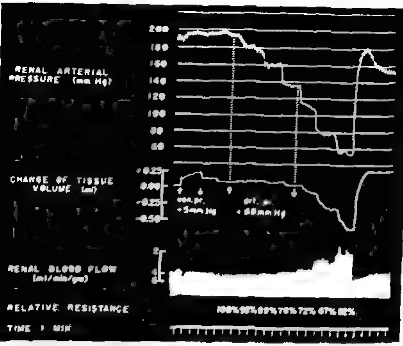

Fig 4 Effect of changes in renal arterial pressure on renal blood flow and renal volume as recorded from decapsulated kidney enclosed in a plethysmograph The two pairs of arrows below the volume recording indicate the periods during which the venous pressure was increased 5 mm Hg and the arterial pressure changed 60 mm Hg The resistance is given for each pressure level as the per cent of the resistance at the first pressure level

Effect of renal decapsulation on renal blood flow autoregulation

Fig 3 is an actual recording from an experiment in which the renal capsule had been entirely removed from the left kidney without causing any bleeding from its surface while the vasomotor nerve supply to both kidneys was left intact The upper flow recording was obtained from the decapsulated left kidney and the lower recording from the right kidney whose capsule remained intact This record is typical of the results obtained from 3 animals and shows that although there was a slightly higher flow per gram of renal tissue in the decapsulated kidney at each pressure level — a fact that will be discussed below — the autoregulatory ability at arterial pressures above 60– 90 mm Hg was not significantly influenced by the decapsulation At lower pressures the capsulated kidney showed a slightly better autoregulatory capacity a phenomenon that will also be discussed below

The second section of Fig 3 demonstrates that blood flow autoregulation is largely abolished in both kidneys when they are exposed to the same degree of a fairly extensive constrictor fibre discharge induced by the Cushing reflex. However the flow resistance within the decapsulated kidney now becomes somewhat more decreased by arterial pressure increases suggesting a more

profound passive distension of its vascular bed as compared with the capsulated kidney This difference too will be further discussed below

Since the autoregulatory ability of the kidney was not significantly influenced by decapsulation at least not at pressures above 80—90 mm Hg 5 additional experiments were performed in which the decapsulated left kidney was placed in a small air filled plethysmograph In this way the volume changes resulting from changes in arterial and venous pressures could be compared The results from these experiments are represented by the actual recording from a typical experiment in Fig 4 After establishing an isovolumetric state the venous pressure was increased 5 mm Hg (from 11 to 16 mm Hg) and the volume of the kidney then increased by 0 1 ml When the arterial perfusion pressure was changed however neither the volume of the kidney nor the slope of the volume curve changed more significantly until the arterial pressure was reduced to such low levels that blood flow autoregulation tended to vanish In other words as long as the mechanisms causing a really efficient autoregulation seemed to be operating the volume of the kidney was largely unaffected by changes in arterial perfusion pressure It was necessary to reduce the arterial pressure from 195 mm Hg to 135 mm Hg in order to cause nearly as much change in renal volume as a 5 mm Hg change in venous pressure produced This is less than half the change in renal volume that would be expected to occur if there was no concomitant change in the resistance proximally to the tubular capillaries When however the autoregulatory mechanism became less efficient and blood flow began to change substantially with each change in pressure the concomitant changes in renal volume became more evident This suggests that at least in the higher pressure range a change in arterial pressure was fairly effectively balanced by a change in the flow resistance proximally to the tubular capillaries It should also be mentioned that in some of these experiments where the preparation slowly deteriorated the blood flow autoregulation vanished gradually over the whole range of arterial pressures and then the tissue volume changed more and more with changes in arterial pressure This finding indicates that by now the active vascular adjustment within proximal parts of the vascular circuit had vanished so that arterial pressure changes were transmitted over to the capacitance side of the vascular circuit

Discussion

The purpose of this series of experiments was to study the autoregulation of renal blood flow in several different situations using the same animal and the same experimental methods throughout as far as was possible It was hoped that in this way some of the controversial questions concerning this subject could be answered Experiments by other investigators de Wardener and Miles (1952) and Thurau and Kramer (1959) for example have indicated that when the renal blood flow is reduced by vasoconstriction the efficiency of

the mechanisms causing autoregulation is reduced. This has been confirmed in the present study by demonstrating that renal vasoconstriction resulting from noradrenaline infusion can abolish the autoregulatory ability of the kidney. In addition, a similar effect was obtained by activation of the renal vasoconstrictor fibres, the elimination of flow autoregulation being more complete the more intense the neurogenic vasoconstriction.

This suggests that although the local mechanisms responsible for renal autoregulation are operating efficiently as long as the kidney is slightly or not at all influenced by the sympathetic system, they can be completely overpowered when the sympathetic discharge to the renal vessels becomes more extensive. In such situations, which occur both in some physiological and pathophysiological circumstances, the renal vessels can be expected to react more like passive elastic tubes to arterial blood pressure changes.

If thus the mechanisms responsible for local control of renal blood flow imply mainly an 'active' adjustment of the precapillary resistance vessels, notably of the afferent arterioles as has been suggested, the vasoconstriction resulting from the increased vasomotor fibre activity or from blood borne catechol amines must be imagined to overpower these local adjustments of vascular tone in some way. If, on the other hand, renal blood flow autoregulation were merely a passive phenomenon depending on a pressure induced distension of the capacitance vessels, which increase tissue pressure and thus curtails the effective pressure head increase, then the vasoconstriction must in some way be able to minimize such a tissue pressure increase. This is theoretically quite possible if it is assumed that the vasoconstrictor fibres contract the renal capacitance vessels as well, thus unloading the renal capsule and decreasing the extent of tissue pressure rise for a given rise in arterial pressure. However, the development of such changes in tissue pressure must at least to a considerable extent depend on the presence of the renal capsule, but it was here found that removal of the capsule on one of the two kidneys did not significantly alter its ability to autoregulate its blood flow as compared with its normal counterpart, at least not at moderate or high arterial pressures.

It might then be suggested that the decapsulated kidney is still rigid enough to cause considerable tissue pressure increases when arterial pressure is increased, hence still being able to produce flow autoregulation by an entirely passive mechanism. However, this seems unlikely because when both the pressure flow relationship and the kidney volume were recorded in decapsulated kidneys, it was found that the renal volume did not change appreciably as a result of even big changes in arterial pressure as long as the mechanisms responsible for autoregulation were performing efficiently, while even small venous pressure changes produced appreciable renal distensions. Only when the arterial pressure was varied at such low levels that renal blood flow autoregulation normally began to fail, or when normal autoregulation tended to vanish in the course of an experiment, the renal volume changed considerably along with

arterial pressure changes This fact suggests that in the autoregulating kidney the changes in arterial pressure over a wide range are normally prohibited from affecting the capacitance vessels more significantly by contrary-directed active changes in vascular smooth muscle activity occurring proximally to the tubular capillaries and presumably to the glomerular capillaries too

It then follows that the abolishment of flow autoregulation by e g a neurogenic vasoconstriction must be ascribed mainly to an 'extrinsic' interference with this local adjustment of vascular smooth muscle activity Possibly the powerful extrinsic drive exerted by the released adrenergic transmitter so dominates the vascular smooth muscles that more subtle local regulatory factors become entirely overruled It is true that the steadily operating baro- and chemoreceptor reflex adjustments of the cardiovascular system have surprisingly little effect on the renal vessels leaving this important vascular circuit largely undisturbed by most centrally governed adjustments However the renal vessels are richly supplied with vasoconstrictor fibres and there are certainly situations when these fibres become intensely activated For example in connection with the hypothalamically integrated defense alarm reaction (c f Feigl Johansson and Lofving 1962) intense pain fibre activation (c f Johansson 1962) or when other influences like asphyxia add to the excitatory drive upon the vasomotor centre it has been observed that an intense sympathetic discharge is induced to the renal vessels too (cf Folkow Johansson and Lofving 1961) It may then be expected that e g trauma causing pain and enough blood loss to lower blood pressure may profoundly reduce renal blood flow firstly because of the neurogenic constriction *per se* secondly because the renal vessels now no longer are able to compensate for the pressure drop by way of the autoregulatory mechanism

Even though the present experiments strongly suggest the presence of a dominating active mechanism in producing renal blood flow autoregulation present as long as the vasoconstrictor fibres to the kidney are not more extensively activated they do not rule out the importance of the capsule-dependent passive mechanism On the contrary several of the present findings do indicate that this passive mechanism is of some physiological significance though being in most situations overshadowed by the active component However in certain situations such a passive mechanism seems to add more appreciably to the autoregulation phenomenon When for instance a normally capsulated and an acutely decapsulated kidney were compared in the same animal it was observed *firstly* that the blood flow level was always slightly lower per unit weight in the normal kidney *secondly* that the normal kidney autoregulated somewhat better in the lower pressure range below [illegible] mm Hg and *thirdly* that the normal kidney changed its flow resistance somewhat less in a passive-elastic fashion when the smooth muscles of the renal vessels were dominated by e g vasoconstrictor fibre activity These findings suggest that the presence of the renal capsule creates a fairly high tissue pressure which

inevitably at all pressure heads curtails to some extent the effective driving pressure over the renal vascular bed and hence its blood flow too. Normally the active autoregulatory mechanism seems to keep the tissue pressure fairly constant until it ultimately fails in this respect at lower arterial pressures. Then, however, a passive elastic recoil of the capacitance side of the renal vascular bed becomes apparent, in its turn unloading the tissue pressure and by this effect helping to minimize the drop in effective pressure head at further drops in arterial pressure. A passive autoregulatory mechanism as suggested by Hinshaw *et al.* (1959) thus seems to operate at lower pressures, then taking over from the by now failing active mechanism.

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Electric Fish (Gymnotus carapo) as a Tool for Bioassay of Methylphenidate and Chlorpromazine

By

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Abstract

Holmstedt B W Krivoy and D Kroeger *Electric fish (Gymnotus carapo) as a tool for bioassay of methylphenidate and chlorpromazine* Acta physiol scand 1964 *61* 177—181 — *Gymnotus carapo* an electric knife fish produces a continuous spontaneous discharge the rate of which is subject to modulation, when the fish is disturbed Free swimming knife fish of this species were subjected to the action of chlorpromazine and methylphenidate while the discharge was detected by electrodes in the corners of the aquarium and recorded on magnetic tape Methylphenidate increased and chlorpromazine decreased the frequency The log dose response curves for both compounds presented a straight line relationship The usefulness of the technique for bioassay of compounds acting on the central nervous system is discussed briefly

Previous reports from these laboratories (Krivoy *et al* 1962 Krivoy Lane and Kroeger 1963 1964) have indicated the possible use of the electric knife fish *Eigenmannia virescens* as a subject for the bioassay of neurotropic drugs Bioassay was based on the fact that although these fish discharges spontaneously at a constant frequency the amplitude of the emitted signal is modulated at a rate which is related to mechanical stimulation or to drug administration Analysis of amplitude modulation, a relatively difficult electronic technique was made more difficult since the fish had to be free swimming *Gymnotus carapo* another electric knife fish produces a continuous spontaneous discharge the rate of which is subject to modulation when the fish is disturbed (Grundfest 1957) Since frequency modulation is a far simpler phenomenon to measure this fish was tested to determine whether or not it is a more suitable object for bioassay than *E virescens* This paper is a report on the response of *G carapo* to chlorpromazine and methylphenidate

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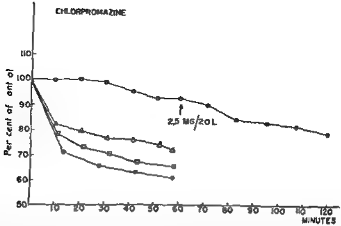

Fig 1 The influence of 5 different concentrations of chlorpromazine on the rate of discharge of *G carapo* Each line is obtained from a single experiment Each point represents the mean rate (cycles per second) for approximately 10 min
Chlorpromazine ○—○ 1 25 × 10^{-}[illegible] g/ml
◐—◐ 1 38 × 10^{-}[illegible] g/ml
△—△ 1 25 × 10^{-}[illegible] g/ml
□—□ 3 12 × 10^{-}[illegible] g/ml
●—● 1 25 × 10^{-}[illegible] g/ml

Methods and materials

G carapo produces a continuous discharge of approximately 40 triphasic pulses per second If the fish is kept in absolute quiet for a prolonged period of time this rate may be reduced to 30 pulses per second and if the fish is disturbed it may be increased to 70 pulses

G carapo differs from *E virescens* in that its electrical activity is diminished in light and increased in the dark Furthermore stabilization of *G carapo* takes a considerably longer time once it has been disturbed than does stabilization of *E virescens* Consequently conditions were standardized by precise replication of equilibration periods and lighting conditions between different experiments

Two days prior to use one fish was isolated in an individual 20 l aquarium On the day of the experiment the aquarium was divided into two unequal compartments by means of a plastic grid The fish was kept in the smaller compartment the size of which was governed by the size of the fish The fish was free swimming and not restricted by the plastic grid any more than was necessary to record its electrical discharge

A pair of silver electrodes was then placed in the same compartment as the fish The electrodes were placed in opposite corners of the compartment so as to facilitate recording of the potentials generated by the fish regardless of amplitude modulation resultant to modification of signal output or regardless of orientation of the fish generator in the conducting medium The sides of the aquarium were then covered with card board and the top with red plastic leaving enough space between the plastic cover and the sides of the aquarium for the introduction of the drugs being tested

The electrical activity of the fish was recorded after suitable amplification on magnetic tape and monitored continuously by means of a cathode ray oscilloscope

The electrical activity of the fish generally became stable two to three hours after it had been prepared for recording A control period was then recorded on tape for one hour (with the exception of one experiment in which a 30 min period was used)

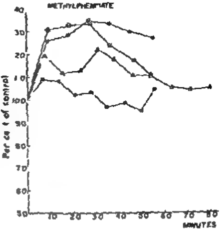

Fig 2 The influence of 5 different concentrations of methylphenidate on the rate of discharge of *G carapo* Each line is obtained from a single experiment Each point represents the mean rate (cycles per second) for approximately 10 min Methylphenidate ○—○ 2 5 × 10⁻ g/ml
△—△ 2 5 × 10⁻ g/ml
□—□ } 2 5 × 10⁻⁴ g/ml
●—● }

At the end of the control period the drug being tested was pipetted into the aquarium After a suitable period of time based on observable alterations in the monitored signal (5 min in the case of methylphenidate 20 min in the case of chlorpromazine) the potentials were again recorded for one hour At the end of that time the experiment was discontinued and the fish was transferred to a drug free aquarium

For analysis, the signals recorded on tape were amplified filtered (to remove sixty cycle) and integrated (time constant 11 msec) Thus each triphasic potential was reduced to a single potential regardless of the orientation of the fish relative to the recording electrodes The integrated potential was amplified further filtered (to remove any additional sixty cycle interference which had been introduced during amplification) and converted into a square wave by means of a square wave amplifier The number of square waves was then counted on a Nuclear Chicago Ultrascaler and the time required to produce 2 000 such counts was printed on a Nuclear Chicago Printer Recycling of this system at the end of 2 000 counts required 15 sec During this time no information was recorded

The pH of the aquarium was maintained at 8 2 and the temperature at 25 C The water was pre treated with EDTA 10 mg/l because of the sensitivity of these fish to bivalent ions Aeration provided adequate mixing of the drug in the aquarium

Results

The administration of chlorpromazine and methylphenidate to *G carapo* resulted in alterations in the direction as well as the magnitude of the frequency of the spontaneous electrical discharge Chlorpromazine which causes central nervous system depression in mammals tended to slow the frequency of discharge of *G carapo* whereas methylphenidate a central nervous system stimulant increased the frequency of the spontaneous discharge

Fig 1 illustrates the effect of chlorpromazine expressed as the percent of the control period plotted against time The smallest dose 0 0125 mg/l (1 25 × 10⁻⁴ g ml) did not produce a marked change in the discharge frequency of the fish until approximately 50 min after drug administration

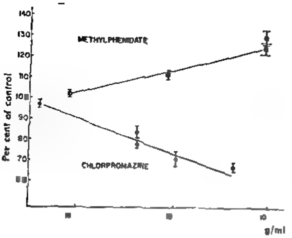

Fig 3 The dose response curve of *G carapo* to chlorpromazine and to methylphenidate Each point is a single experiment and is the mean of the post drug discharge rate compared to each control The SE is given for each experiment

In one particular experiment appearing in Fig 1 and only in one experiment an additional amount of chlorpromazine (2 5 mg) was added after the first hour This additional amount of drug increased the total concentration of chlorpromazine in the aquarium to $1\ 3 \times 10^{-7}$ g/ml and caused a further slowing of the frequency Other experiments in which chlorpromazine was given in final concentrations of $1\ 25 \times 10^{-7}$ $3\ 12 \times 10^{-7}$ and $1\ 25 \times 10^{-6}$ g/ml respectively caused increasing degrees of slowing of the discharge frequency

Methylphenidate produced an increase of the frequency of the spontaneous discharge Fig 2 illustrates the acceleration of the rate of discharge produced by 3 different doses of methylphenidate Each of these three different concentrations caused a significant increase in the rate of discharge during the first 10 min period For all doses except the smallest the effect persisted for the remainder of the 60 min period of observation It may also be seen that there is good agreement when the actions of methylphenidate are compared in different experiments It is to be noted that methylphenidate did not produce a sustained action as did chlorpromazine The reason for this more rapid disappearance of methylphenidate activity was not investigated

Fig 3 illustrates the log dose response curve obtained both for methylphenidate and for chlorpromazine This figure was obtained by determining the percent change when the mean discharge rate (cycles per second) during the entire control period was compared with the mean discharge rate during the hour of recording (see Methods) following the administration of each dose of the drug

Discussion

It is obvious that this technique may be useful in the qualitative and quantitative evaluation of drugs such as chlorpromazine and methylphenidate Based on the responses of *E virescens* to other drugs (Krivoy et al 1962 1963

1964) it is likely that this technique will prove valuable for the evaluation of the actions of a number of different compounds such as neurotropic polypeptides narcotic analgetics and anesthetic agents The present bioassay would probably be more useful if behavioral tests such as conditioning were performed in conjunction with the drug studies

The bioassay as stated is simple to use relatively inexpensive and extremely sensitive Furthermore the information obtained seems to be readily quantitatible

Like most bioassays one cannot off hand use the technique to evaluate the mechanism of action of drugs However it is reasonable to assume that even this may be possible if one were to undertake appropriate experimental conditions

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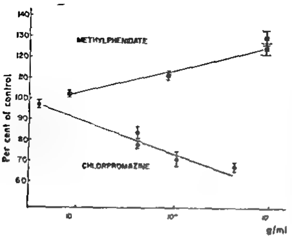

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Discussion

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line is important more as a supplementary defence against cold The response of the adrenal cortex to cold stimuli appears to be more variable and its importance in temperature regulation is therefore less well understood (cf Heroux 1960 and Knigge 1960)

In most studies of hormonal cold defence mechanisms a lowering of the external temperature causing little or no change in the core temperature has been used as the experimental stimulus The relative importance of central thermodetectors as compared to that of peripheral cold receptors in the control of non shivering thermogenesis therefore has been difficult to assess It may be expected however that thermal changes in the preoptic region (the heat loss center) being the site of central "warm detectors" (Magoun *et al* 1938 C v Euler 1950 Nakayama et al 1963) influence the activity of the sympathico adrenomedullary and the pituitary adrenocortical systems Such an influence has already been shown to exist in regard to another hormonal mechanism involved in heat production the pituitary thyroid system (Andersson *et al* 1963) It was shown that local cooling of the heat loss center causes thyroid activation whereas the corresponding warming inhibits the thyroid activation normally seen during a general cold stress The following experiments were therefore undertaken to study whether similar central cooling or warming would affect the production of adrenaline and noradrenaline as indicated by changes in urinary catecholamine excretion in addition in some experiments the influence on adrenocortical function was studied using the plasma cortisol level as an indicator

Methods

Adult female goats weighing 30 to 35 kg were used The urinary excretion of catecholamines was followed in four animals during central cooling (15 expts) and in three goats during central warming (3 expts) Plasma cortisol determinations were made in 6 expts involving central cooling and in two involving central warming

The technique of central cooling was essentially the same as that used in previous experiments (c f Andersson *et al* 1963) i e cold water perfusion of silver thermodes permanently implanted medially in the preoptic/anterior hypothalamic region This technique permitted repeated local cooling of the rostral hypothalamus over long periods of time in animals maintained with no additional restraint in their accustomed environment The most thoroughly studied goat in this series of experiments was centrally cooled in 22 expts over a 9 month period In 10 of these experiments the urinary excretion of catecholamines was followed In each experiment central cooling was started *insofar as possible at the same time of the day and was continued for 2–3 hours* Room temperature ranged between 15 and 21 C but was close to 18 C in most experiments

Local warming of the preoptic anterior hypothalamic region was accomplished either by warm water perfusion of thermodes also used for cooling or in one of the present animals by radio-frequency (RF) warming between two bilaterally implanted silver plates (Andersson et al 1963) During the pre warming period the room temperature was maintained near 20 C but was rapidly lowered to between [illegible] C by opening the windows in winter time after commencement of central warming The room temperature was then maintained at this low level throughout the experiment

Table I The effect of preoptic/anterior hypothalamic cooling on urinary excretion of catecholamines

	Average duration (min)	Adrenaline (ng/min)		Noradrenaline (ng/min)	
Pre-cooling period	120	1 7 ± 0 2 (13)	P < 0 01	6 3 ± 0 3 (13)	P < 0 05
Cooling period	140	3 7 ± 0 3		9 3 ± 0 7	
			P < 0 01		P < 0 05
Post-cooling period	160	1 9 ± 0.2		6 3 ± 0 5	

All values are the mean ± SEM Number of experiments in parentheses P = Statistical significance between indicated means determined by Student s *t* test when the SEM was assumed to be homogenous, otherwise the Cochran Cox *t* test was used

In order to avoid the excitatory effect of catheterization urine was collected only when normally voided Since goats micturate in a characteristic position and empty the bladder completely each time the collection of clean and complete urine samples was not difficult In order to produce a mild increase in urine secretion the goats were usually given a liter of milk water mixture about one hour before start of urine collection Each sample of urine was acidified immediately after being voided and its content of adrenaline and noradrenaline was estimated fluorimetrically according to the procedure described by Euler and Lishajko (1961) In some experiments jugular blood was drawn at intervals for determination of plasma cortisol according to the description of Peterson Karrer and Guerra (1957)

In the experiment involving adrenergic blockade 10 mg/kg of phenoxybenzamine (Dibenzyline hydrochloride Smith Kline & French) was infused into the jugular vein over a period of one hour

In some experiments involving central cooling thyroid activity was studied in parallel to the excretion of catecholamines The method was the same as used in previous experiments (cf Andersson et al 1963) Blood sugar was determined according to the method of Somogyi (1945)

Results

A Central Cooling

1) Urinary excretion of catecholamines In all 4 animals local cooling of the preoptic/anterior hypothalamic region was found to cause an increase in the urinary excretion of catecholamines The mean excretion of adrenaline and noradrenaline before during and after central cooling in 13 expts is shown in Table I As can be seen the relative increase in adrenaline excretion during the cooling period was considerably greater than that of noradrenaline (117 % *vs* 48 % increase) There was also a tendency for a reduction of the noradrenaline response the more frequently central cooling was performed in the same animal Indeed in a few experiments central cooling caused practically no increase in noradrenaline excretion although the excretion of adrenaline still

Fig 1 *Two experiments in the same goat showing the catecholamine response to local cooling of the preoptic anterior hypothalamic region*

Left An experiment after a 40-day period during which the animal had not been centrally cooled

Right A similar experiment when central cooling had been performed repetitively (10 times) during a 30-day period

Note the equivalent rise in core temperature but the difference in noradrenaline response suggesting that repetitive central cooling induces cold acclimation

Room temperature in both experiments 16 to 17 C.

Body weight of the goat 35 kg

increased This was most clearly demonstrated in the goat used for 22 cooling experiments over 9 months In this animal there were two periods of 40 and 85 days respectively when no central cooling was performed In the initial experiments performed after these long intervals the noradrenaline response to central cooling was much more pronounced than when the cooling had been performed at briefer intervals (Fig 1 A compared to Fig 1 B)

2) Plasma cortisol level In 6 expts plasma cortisol was determined at hourly intervals before during and after central cooling The first sample during the cooling periods was taken one half to one hour after the start of central cooling Great variations (from 0 to 10 μg/100 ml plasma) were found among the samples but with no significant trend of an increased plasma cortisol level during the periods of central cooling

3) Other effects Other thermoregulatory and alimentary effects of central cooling were essentially the same as observed in previous experiments (cf Andersson Gale and Sundsten 1963) Thus cooling of the heat loss center caused peripheral vasoconstriction initial shivering thyroid activation and a rise in core temperature of about 2 C Further in the previously fed animals it induced a strong urge to eat within 5 min of central cooling Repetitive cooling of the preoptic anterior hypothalamic region over long periods of time induced certain changes in the animals which are normally seen during acclimation For example the fur growth was stimulated such that repeatedly centrally cooled animals developed noticeably thicker coats than other goats maintained under identical external conditions There also occurred a progressive

increase in plasma protein bound iodine level and a gradual weakening of the shivering response to central cooling

4) Influence of an adrenergic blockade on the response to central cooling Since it seemed to be of interest to study to what extent an adrenergic blocking agent would affect the excretion of catecholamines and other responses to central cooling one of the goats was given 10 mg/kg of phenoxybenzamine via a one hour i v infusion The adrenergic blockade in itself did not change the body temperature of the goat but caused a conspicuous elevation of catecholamine excretion During a period of urine collection between one and two hours after the infusion the excretion of adrenaline and noradrenaline reached its maximum 368 ng/min for adrenaline and 274 ng/min for noradrenaline (about 120 and 40 times respectively above normal levels for this goat) Thereafter catecholamine excretion gradually declined such that 20 hours after the infusion the excretion rate for both adrenaline and noradrenaline was only 10 times normal During a two hour period of local cooling of the preoptic/anterior hypothalamic region at this stage there was slight but definite increase in urinary excretion of both adrenaline and noradrenaline followed by a resumption of the decline after cessation of central cooling During this period of central cooling shivering was much more intense and of longer duration than in corresponding experiments in the same animal without previous administration of phenoxybenzamine The rise in core temperature was of the same order (2 C) as in previous experiments involving central cooling Further this goat which after the administration of phenoxybenzamine had completely lost interest in food started to eat hay eagerly after a few minutes of central cooling and continued eating the 2 hours the cooling lasted

B Central Warming

1) Urinary excretion of catecholamines To study whether a high temperature in the "heat loss center would counteract an increase in catecholamine excretion during cold exposure and general hypothermia the preoptic/anterior hypothalamic region was locally warmed in 3 goats Immediately after start of central warming the room temperature was rapidly lowered from 20 C to between 2 and 5 C In the RF warmed animal the rostral hypothalamus was maintained at 42 C In the 2 other goats the temperature 2 mm lateral to the lateral surface of the thermodes was maintained close to 41 C by warm water perfusion of the thermodes Central warming caused the expected activation of physical heat loss mechanisms (peripheral vasodilatation polypnea) and at the same time inhibited shivering As result there was a steady decline of the core temperature during central warming in the cold environment In 2 of the goats central warming was stopped when the core temperature had fallen to about 36 C In the third animal a two-hour period of central warming lowered the core temperature to 31 C Within 2 min after cessation of central warming

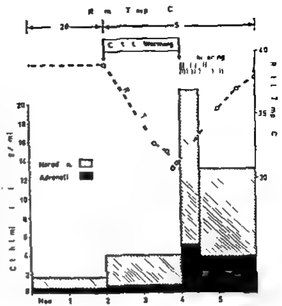

Fig 2 *Inhibition of the sympathico-adren medullary response to cold by local warming of the preoptic anterior hypothalamic region*
Local warming of the heat loss center simultaneous with sudden exposure of a goat (b w 30 kg) to external cold (5 C) elicited peripheral vasodilatation polypnea and a gradual fall of core temperature to 31 C Despite this considerable hypotherm a and the cold environment catecholamine excretion was only slightly elevated so long as central warming lasted Upon cessation of this warming strong shivering developed associated with a considerable increase in the excretion of both adrenaline and noradrenaline

the goats were shivering vigorously and the core temperature started to rise again During the rising phase of body temperature the blood sugar increased from a normal value (about 50 mg/100 ml) just before cessation of central warming to over 100 mg/100 ml one half to one hour later At this stage the urinary excretion of catecholamines especially that of noradrenaline was considerably higher than before and during central warming In the two goats which developed only moderate hypothermia the noradrenaline excretion during the first post warming period increased 2 1/2 times whereas only a small increase in adrenaline excretion was observed In the animal which developed a more severe hypothermia during central warming in the cold (31 C) the urinary excretion of both adrenaline and noradrenaline was 7 times higher during the first 30 min after cessation of central warming than it had been during the actual warming period During earlier experiments involving central cooling at room temperatures round 18 C the urinary excretion of catecholamines never reached such a high level in this animal The experiment is illustrated in Fig 2

2) *Plasma cortisol level* Determinations of plasma cortisol in two of the experiments with central warming gave no conclusive results The highest levels in both goats (14 and 20 μg/100 ml plasma respectively) were found during the first hour after cessation of central warming in the cold but occasional values of almost the same magnitude were also found among the pre warming and warming samples

Discussion

Leduc's (1961) recent investigation has contributed greatly to the elucidation of the importance of the sympathico adrenomedullary system in non shivering heat production and in acclimation to cold His studies in the rat show that the immediate response to moderate cold exposure (3 C) is an increased release of noradrenaline from the adrenergic nerve endings An accelerated release of adrenaline from the adrenal medulla appears later as a secondary response during prolonged cold exposure However, during acute exposure to more severe cold (— 7 C) the adrenal medulla responds much more readily with increased adrenaline release This finding is in accordance with earlier observations in unanesthetized dogs (Wada Seo and Abe 1935) In the latter species a more marked increase in adrenaline secretion seems to first occur when the cold stimulus is severe enough to cause a fall in body temperature Leduc s studies further show that on prolonged exposure to cold the noradrenaline excretion remains elevated although slowly declining whereas adrenaline excretion reaches its maximum only after a week and then rapidly decreases When cold acclimated rats are re exposed to a low environmental temperature they maintain their body temperature more easily and excrete less catecholamines than do warm acclimated rats under the same conditions This seems to be explained by the earlier observation (Hsieh and Carlson 1957 Hsieh Carlson and Gray 1957) that the calorigenic effect of adrenaline and especially of noradrenaline increases during cold acclimation Since the calorigenic action of noradrenaline is potentiated by thyroxine it is likely that the pituitary thyroid system participates in this acclimation response (cf Carlson 1960)

But the sympathico adrenomedullary system is obviously also intimately related to other thermoregulatory mechanisms Thus the maintenance of a relatively constant body temperature in spite of wide variations in the temperature of the external environment demands a complex interplay between all neural and hormonal thermoregulatory factors In addition to peripheral cold receptors central warm detectors have been shown to play an essential role in the control of body temperature The classical experiments of Magoun *et al* (1938) focused interest on the preoptic/anterior hypothalamic region as a site for such warm detectors More direct evidence for the presence in the rostral hypothalamus of elements specifically sensitive to heat has been provided by electrophysiological recording of neuronal activity in this part of the brain (C v Euler 1950 Nakayama *et al* 1963) This heat loss center not only serves to activate physical heat loss mechanisms (peripheral vasodilatation sweating and polypneic panting) In addition it apparently also exerts a brake even at normal body temperature on neural cold defense mechanism (Hemingway *et al* 1940 Andersen Andersson and Gale 1962) and on at least one hormonal cold defense mechanism the pituitary thyroid system (Andersson *et al* 1963)

The aim of the present study was to investigate further the role played by these central warm detectors in the control of other hormonal cold defense

mechanisms of importance in heat production Local cooling of the rostral hypothalamus (likely to block or at least markedly reduce the activity of the heat loss center) was found to cause increased urinary excretion of catechol amines Local warming of the same region (increasing the activity of central warm detectors) was found to prevent the activation of the sympathico adrenomedullary system expected during a cold stress (cold exposure combined with a gradual lowering of the body temperature) These observations suggest that central warm detectors in the preoptic/anterior hypothalamic region counteract the stimulatory effect on the sympathico adrenomedullary system of an increased inflow from peripheral cold receptors It is of considerable interest that although the immediate response to cold exposure is predominantly an increased release of noradrenaline from adrenergic nerve endings (Leduc 1961) the central cooling most consistently caused an increase in adrenaline excretion Accordingly on cessation of central warming in the cold when profound hypothermia (31 C) had developed there was a marked increase in both adrenaline and noradrenaline excretion (Fig 2) At a milder degree of hypothermia (36 C) however there was still a marked increase in noradrenaline excretion on cessation of central warming, whereas relatively little increase of adrenaline excretion was observed Recent microelectrode studies indicate that central warm detectors in the preoptic region are slightly active at brain temperature as low as 32 C but that their spontaneous discharge increases with increase in brain temperature to 41° C (Nakayama et al 1963) One must therefore consider the possibility that the heat loss center still exerts some inhibitory tone on cold defense mechanisms at temperatures considerably below normal level It may be that a relatively high degree of activity in this center is needed to counteract the stimulatory effect of peripheral cold on adrenergic nerves (noradrenaline secretion) whereas the adrenal medulla (adrenaline secretion) is more readily inhibited This may afford an explanation why in the present experiments the direct cooling of the rostral hypothalamus (an almost complete inhibition of central warm detectors) caused an increase in adrenaline excretion and also why in the warming experiments a considerable rise in adrenaline excretion upon cessation of central warming occurred only when marked hypothermia had developed For the further evaluation of the relative importance of peripheral cold receptors and central warm detectors it would be of particular interest to compare the catecholamine response to central cooling in a cold and in a warm environment

In view of Leduc s (1961) findings the decrease in noradrenaline response on repetitive central cooling suggests an acclimation to cold The gradual increase in basal thyroid activity the diminishing shivering response and the intensified fur growth support this concept It would then mean that cold acclimation may occur in spite of no lowering of the external temperature provided the central warm detectors are repeatedly inactivated It is in

agreement with the observation of Adolph and Richmond (1956) that cold acclimation follows more rapidly on induction of core hypothermia than on simple exposure to cold

A marked increase in noradrenaline excretion has been observed been observed following the administration of phenoxybenzamine in rats (Schapiro 1958 Leduc 1961) In the goat both adrenaline and noradrenaline excretion rose to very high levels after this drug was given Since this increase in catecholamine excretion was much greater than ever observed during central cooling it can be concluded that such cooling by no means exhausts the catecholamine production Further, there appeared to be a striking difference between the effect of an adrenergic and a ganglionic blockade on the response to central cooling The adrenergic blockade did not prevent the temperature ri e during local cooling of the rostral hypothalamus and shivering became markedly accentuated A ganglionic blockade, on the other hand was recently observed to prevent the rise in temperature during central cooling and to reduce the shivering response (Andersson *et al* 1964)

In contrast to the consistent response of the thyroid and the sympathico adrenomedullary system the experimental data obtained so far when studying blood cortisol level in the goat are impossible to evaluate The values showed great variation, but without any clear cut correlation to the stimulus applied whether central cooling or warming was used Chowers *et al* (1963) have recently observed an elevated blood cortisol level within the first half hour after commencement of preoptic cooling in the dog It cannot be excluded that a similar response to central cooling may also have been present in the goats although not detected in the present study due to relatively infrequent sampling It is hoped that the combined determination of plasma cortisol and urinary corticosteroid excretion during experiments of this kind may help to clarify a possible interaction between the heat loss center and the pituitary adrenocortical system

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Acta physiol scand 1964 61 192—194

From the Department of Pharmacology University of Goteborg Sweden

Influence of Nethalide on the Phosphorylase Activating Effects of Adrenaline and Isoprenaline in Experiments on Isolated Rat Diaphragm

By

Ella Mohme Lundholm and Nils Svedmyr

Received 5 November 1963

Abstract

Mohme Lundholm E and N Svedmyr *Influence of nethalide on the phosphorylase activating effects of adrenaline and isoprenaline in experiments on isolated rat diaphragm* Acta physiol scand 1964 *61* 192—194 — Nethalide at a concentration of 10^{-6} totally inhibited the phosphorylase activating effect of L-adrenaline or L isoprenaline at a concentration of 10^{-6} in experiments on isolated rat diaphragm

Nethalide is a recently introduced adrenergic beta receptor inhibitor which has been found to be capable of blocking the relaxing and the cardiostimulatory effects of adrenaline and isoprenaline as well as certain metabolic effects of those catechol amines (Black and Stephenson 1962 Dornhorst and Robinson 1962) At the concentration tested, however it failed to block the hyperglycemic action of adrenaline (Pilkington *et al* 1962) In the light of the close correlation between the effects of certain catechol amines on beta receptors and their effects upon the carbohydrate metabolism (Lundholm and Mohme Lundholm 1960) this result was somewhat surprising The hyperglycemic effect of adrenaline is attributed to activation of the enzyme phosphorylase which catalyzes the reaction glycogen glucose 1 phosphate (review Rall and Sutherland 1960) We have conducted experiments to determine whether nethalide influences the phosphorylase activating effects of adrenaline and isoprenaline *in striated muscle* — In the latter tissue two forms of phosphorylase occur the *a* form being active even in the absence of AMP the *b* form only in the presence of AMP Catecholamines stimulate the transformation of phosphorylase *b* ⟶ *a* (Rall and Sutherland 1960) — Nethalide was found to block completely the phosphorylase activating effects of both adrenaline and isoprenaline

Table I Effect of nethalide on stimulation of phosphorylase activity by L-adrenaline and L-isoprenaline in isolated rat diaphragm Total phosphorylase activity (T) was determined in the presence of 0.001 M AMP and is recorded in mg inorganic phosphate liberated per g muscle per min Phosphorylase a activity is expressed as the amount of phosphate liberated without AMP in per cent of that liberated with AMP P = probability that the effect was due to chance n = number of tests

	Control value		Change from control after adrenaline or isoprenaline		Change from control after nethalide	Change from control after nethalide + adrenaline/isoprenaline
Drug	a	T	a	T	a	a
Adrenaline (n = 11)	20.5±2.3	0.35±0.04	14.8±2.9 P < 0.001	0.032±0.009 P < 0.01	−3.5±1.4	0.6±2.8
Isoprenaline (n = 8)	22.5±2.8	0.37±0.05	21.4±5.3 P < 0.01	0.083±0.033 P < 0.05	−4.4±3.7	−1.6±5.2

Difference in effect on a as between tests without nethalide and those with nethalide

adrenaline 18.3 ± 3.8 P < 0.001

isoprenaline 25.8 ± 6.5 P < 0.01

Method

A rat was killed by a blow on the head and its diaphragm removed and divided into four equal parts These were weighed then incubated in four test tubes each containing 10 ml Krebs Henseleit glucose free bicarbonate buffer at 37 C, aerated with 5 per cent CO_2 and 95 per cent O_2 giving a pH of 7.40 After 30 min preincubation 0.1 ml diluent for adrenaline (0.9 per cent NaCl + 0.05 per cent Na_2SO_3 + HCl ad pH 3.0) was added to the control 100 µg adrenaline (final concentration 10^{-4}) in 0.1 ml diluent, or an equivalent amount of L-isoprenaline to the adrenaline and isoprenaline preparations respectively 1 mg nethalide (final concentration 10^{-4}) in 0.1 ml diluent to the nethalide preparation and 100 µg adrenaline or isoprenaline plus 1 mg nethalide to the last preparation After 15 min incubation each muscle specimen was extracted in cold with 4 ml of a solution containing 0.02 M NaF plus 0.001 M EDTA by grinding it with a little sand in a mortar Following centrifugation 1 ml of the extract was diluted with 7 ml of a solution consisting of 0.04 M sodium glycerophosphate 0.03 M cysteine hydrochloride and 0.2 M NaF the pH of which had been adjusted to 6.8 Of this solution 0.8 ml was taken for phosphorylase assay *ad modum* Cori Illingworth and Keller (1955) The activity was determined with 0.001 M AMP and without AMP The reaction time was 15 min Prior to use the glycogen had been purified as described in an earlier paper (Mohme Lundholm 1962)

The incubation solution in its entirety (1.6 ml) was used for inorganic phosphate determination *ad modum* Martin and Doty (1949)

Results

It will be seen from the tabulated results that in the adrenaline experiments the proportion of phosphorylase *a* was 20 5 per cent for the control preparations Following addition of adrenaline the proportion of phosphorylase *a* rose to 35 3 per cent, the increase being statistically verifiable The total phosphorylase activity also increased somewhat Nethalide itself had no appreciable effect on the phosphorylase activity though it completely blocked the effect of adrenaline Isoprenaline, too appreciably increased the phophorylase *a* content and somewhat increased the total phosphoryla e activity This effect was likewise inhibited by nethalide Nethalide thus had a conspicuous inhibitory influence on the phosphorylase activating effects of adrenaline and isoprenaline in skeletal muscle

Kennedy and Ellis (1963) have recently found that the adrenergic receptors mediating glycogenolysis in the rat liver and in muscle reacted different to catecholamines Isoprenaline stimulated glycogenolysis in skeletal and cardiac muscle but not in the liver Dichloroisoprenaline inhibited the glycogenolytic effect of adrenaline in skeletal and cardiac muscle but not in the liver This different sensitivity of the receptors in liver and skeletal muscle may explain why Pilkington et al (1962) found no blockade of the hyperglycemic effect of adrenaline by nethalide whereas we found an inhibition of the phosphorylase activating effects of adrenaline and isoprenaline in skeletal muscle by nethalide

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Observations on Antigen-Induced Histamine Release from Rabbit Skin

By

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Abstract

WESTERHOLM, B. *Observations on antigen induced histamine release from rabbit skin.* Acta physiol. scand. 1964. *61.* 195—200. — Antigen 1 mg/ml caused low but significant histamine release from chopped abdominal skin of rabbit. Release was dependent upon pH and temperature. Certain enzyme inhibitors such as allicin (SH group inhibitor) and ninhydrin (NH group inhibitor) blocked release. Reduced glutathione reversed the inhibitory action of allicin. Dinitrophenol and anoxia inhibited release only in glucose free medium. The results suggest that in rabbit the antigen induced histamine release from skin follows a similar pathway to that of the anaphylactic histamine release from rat and guinea pig tissues and occurs after activation of a process requiring energy from oxidative or glycolytic processes.

Histamine has been shown to be released during the anaphylactic reaction in rabbit from such tissues as lung (Dragstedt, Ramirez de Arrelano and Lawton 1940, Schachter 1953), skin, liver and intestine (Schachter 1953) and from platelets (Humphrey and Jaques 1953, 1955, Barbaro 1961a, 1961b).

Very little is known of the mechanism involved during histamine release from rabbit skin in the antigen antibody reaction. In the present investigation histamine release by antigen from rabbit skin was studied *in vitro* under conditions known to influence anaphylactic histamine release from rat and guinea pig tissues. The purpose was to see to what extent the release in rabbit skin resembles that observed in rat and guinea pig tissues during the antigen antibody reaction and in this way to learn something about the release mechanism involved.

Methods

Sensitization procedure

The rabbits used were of both sexes and weighed 1—3 kg. Sensitization was performed by injecting the rabbits intraperitoneally every day for 5 days with 10 ml of a 10 per cent solution of crystallized egg albumin. The animals were used 3 to 4 weeks after the last injection.

Incubation technique

The method employed was the same as that described in an earlier paper for cat skin (Westerholm 1960). Chopped rabbit abdominal skin in portions of 1 g were in

cubated in 25 ml beakers each containing 3 ml of a buffered solution (pH 7 2–7 4) containing NaCl 1 54 × 10^{-1} M KCl 2 7 × 10^{-3} M $CaCl_2$ 9 × 10^{-4} M 10 per cent Sorensen phosphate buffer (Na_2HPO_4 2 H_2O 3 0 × 10^{-3} M KH_2PO_4 3 5 × 10^{-3} M) and antigen Incubation was performed at 37 C for 30 min Oxygen was introduced through cannulae in the stoppers of the beakers In the anoxia experiments nitrogen (99 7 per cent pure) was used instead of oxygen All incubations were run in duplicate

When inhibitors were used the tissue was preincubated with the inhibitor for 30 min Antigen was then added and incubation continued for another 30 min

During incubation for 30 min at 37 C the spontaneous release of histamine was usually too low to be measured even in the presence of inhibitors In the experiments where spontaneous release of histamine was detected release values were corrected for spontaneous release

The variation in histamine release induced by antigen 1 mg/ml in duplicate samples amounted to ± 1 3 per cent of the total histamine content as judged from 23 duplicate tests from different experiments

Assay of histamine

The histamine released into the incubation medium was assayed — after filtering away the skin pieces and boiling the samples — on atropinized guinea pig ileum (atropine sulphate 1 5 × 10^{-6} M) When inhibitors and reactivators were used they were added in equal amounts to the histamine standard Specificity was demonstrated by mepyramine block (mepyramine 5 × 10^{-7} M)

Residual histamine in the skin pieces was extracted by boiling the tissue in N HCl (3 ml/g) for 5 min The extract was neutralized and assayed on guinea pig ileum

Assay of 5-hydroxytryptamine (5 HT)

5 HT in tissue extract was measured spectrophotofluorimetrically according to Bogdanski *et al* (1956)

Materials

) Allicin was prepared from garlic according to Cavallito and Bailey (1944) and was kindly supplied by Mrs I L Thon Department of Pharmacology Karolinska institutet Stockholm Sweden

Other substances used were obtained from standard commercial sources

Results

Histamine and 5-HT content of rabbit skin

The histamine content of abdominal skin from 57 rabbits amounted to 1 9 ± 0 1 μg/g tissue wet weight (range 0 6 — 4 1 μg/g) With the method used no measurable amounts of 5 HT could be found in abdominal skin from four different animals (< 0 01 μg/g tissue wet weight)

Concentration of antigen

The release induced by 1 mg/ml of antigen in 23 different rabbits amounted to 11 8 ± 1 3 per cent (mean ± S E) of the total histamine in abdominal skin Higher doses of antigen were not used because of the interference with histamine assay No significant histamine release could be measured with 0 1 mg/ml of antigen In non sensitized rabbits (4 expts) 1 mg ml of antigen caused no measurable histamine release

Table I Influence of pH on histamine release from rabbit abdominal skin in vitro induced by 1 mg/ml of antigen

Exp no	pH during incubation Initial	Final	Per cent release	pH during incubation Initial	Final	Per cent release
1	7.1	7.2	4	6.1	6.4	Not measurable
2	7.0	7.3	6	6.0	6.4	Not measurable
3	7.0	6.9	21	5.9	6.2	Not measurable
4	7.0	7.2	15	5.9	6.2	2

Table II Influence of temperature on histamine release from rabbit abdominal skin in vitro induced by 1 mg/ml of antigen

Exp no	Release at 0.5° C	Per cent release at 37° C: After rewarming the tissue from 0.5° C	After preheating the tissue at 47.5° C for 15 min
1	Not measurable	8	Not measurable
2	Not measurable	9	Not measurable
3	Not measurable	11	Not measurable

Influence of pH

The effect of pH on antigen induced histamine release from rabbit skin was investigated since it has been shown that anaphylactic histamine release from guinea pig lung is pH dependent (Mongar and Schild 1958, Chakravarty 1960, Chakravarty and Uvnäs 1960). Skin pieces were incubated in standard incubation medium buffered with 20 per cent Sorensen phosphate buffer. In spite of the presence of buffer pH changes were observed during incubation. These made it difficult to determine the pH optimum for antigen induced histamine release. It could be shown however that the release was reduced at low pH (Table I).

Effect of temperature

Antigen induced histamine release from rat skin (Mota and Ishii 1960) and guinea pig lung (Mongar och Schild 1956, 1957a, Mota 1958, 1959, Chakravarty 1960, Chakravarty and Uvnäs 1960) is reversibly blocked at 0.5° C and irreversibly inhibited at 47.5° C. It could be shown that histamine release from rabbit skin by antigen was inhibited at 0.5° C (Table II) but could be restored by rewarming the tissue to 37° C. Preheating of the tissue for 15 min at 47.5° C caused complete inhibition of the antigen induced release at 37° C (Table II).

Table III Inhibition of histamine release from rabbit abdominal skin in vitro induced by 1 mg/ml of antigen

Exp no	Inhibitor	Reactivator	Per cent release: Without inhibitor	With inhibitor	With inhibitor and reactivator
1	Allicin 5×10^{-4} M	—	10	4	—
[illegible]	Allicin 5×10^{-4} M	—	9	Not measurable	—
3	Allicin 5×10^{-4} M	Reduced glutathione 8×10^{-4} M	8	Not measurable	6
4	Allicin 5×10^{-4} M	Reduced glutathione 8×10^{-4} M	26	6	15
5	Allicin 5×10^{-4} M	Reduced glutathione 8×10^{-4} M	10	Not measurable	8
1	Ninhydrin 10^{-3} M	—	14	Not measurable	—
2	Ninhydrin 10^{-3} M	—	20	Not measurable	—
3	Ninhydrin 10^{-3} M	—	11	Not measurable	—
4	Ninhydrin 10^{-3} M	—	14	Not measurable	—
1	Dinitrophenol 5×10^{-4} M	Glucose 5.6×10^{-3} M	13	Not measurable	10
2	Dinitrophenol 5×10^{-4} M	Glucose 5.6×10^{-3} M	18	4	9
3	Dinitrophenol 5×10^{-4} M	Glucose 5.6×10^{-3} M	21	Not measurable	21
1	Anoxia (N_2 induced)	Glucose 5.6×10^{-3} M	9	Not measurable	4
2	Anoxia (N_2 induced)	Glucose 5.6×10^{-3} M	11	Not measurable	10
3	Anoxia (N_2 induced)	Glucose 5.6×10^{-3} M	13	Not measurable	15

Effect of chemical inhibitors and anoxia

Certain enzyme blocking agents such as sulphydryl group inhibitors and amino group reagents have been shown to block antigen induced histamine release from rat and guinea pig lung (Mongar and Schild 1955 1957b Mota 1958 Chakravarty 1959 1960 Chakravarty and Uvnas 1960 Boreus and Chakravarty 1960 Edman and Mongar 1961) and rat skin (Mota and Ishii 1960) In the present investigation allicin (sulphydryl group inhibitor) at a concentration of 5×10^{-4} M and ninhydrin (amino group inhibitor) at a concentration of 10^{-3} M blocked the antigen induced histamine release from rabbit skin (Table III) Lower concentrations of allicin or ninhydrin produced no significant inhibition When the skin was incubated before the addition of allicin for 15 min with reduced glutathione at a concentration of 8×10^{-4} M the inhibitory action of allicin was reversed (Table III)

In the absence of glucose anoxia has been shown to inhibit anaphylactic histamine release from rat and guinea pig lung (Moussatché and Prouvost Danon 1961 Rotschild and Barreto 1961 Chakravarty 1962 Diamant 1962) The antigen induced histamine release from rabbit skin was similarly inhibited (Table III) When the incubation was performed under anoxia but in the

presence of glucose 5 6 × 10⁻³ M no inhibition of the release was observed (Table III)

Provided that there is no glucose in the incubation medium dinitrophenol blocks the antigen induced histamine release from rat diaphragm and guinea pig lung (Rotschild and Barreto 1961) The antigen induced histamine release from rabbit skin was also inhibited by dinitrophenol at a concentration of 5×10^{-4} M in glucose free medium (Table III) If the incubation was performed in the presence of glucose 5.6×10^{-3} M the inhibitory action of dinitrophenol was abolished

Discussion

There is strong evidence that the releasable histamine in the skin of various species originates from the mast cells (Riley and West 1953 Riley 1959) In rabbit skin both the number of mast cells and the amount of histamine were found to be low (Riley 1959) These observations are in agreement with the findings of the present study The histamine content of skin was found to be low as was the amount histamine released by an antigen dose as high as 1 mg/ml In comparison with the amounts of histamine released from platelets during antigen antibody reaction the histamine release from skin in the rabbit seems to be of little importance for the symptoms in the anaphylactic reaction

Schachter (1953) obtained a higher histamine release by antigen from perfused skin of rabbit ear than could be demonstrated in the present investigation from chopped rabbit abdominal skin This may be due to differences in methods and in site of the skin It is known that the histamine content of ear skin is higher than that of abdominal skin in rabbit dog guinea pig and cat (Feldberg and Miles 1953 Feldberg 1956)

Parratt and West (1957) showed by biological methods that the 5 HT content of rabbit skin is very low In the present investigation it was not possible to demonstrate any 5 HT in rabbit skin by spectrophotofluorimetric methods It therefore seems unlikely that any 5 HT of importance is released from rabbit skin during the antigen antibody reaction

From the present investigation it is evident that there are great similarities between histamine release from rabbit skin in the antigen antibody reaction and anaphylactic histamine release from rat and guinea pig tissues Thus low pH high and low temperature sulphydryl and amino group reagents anoxia and dinitrophenol influence antigen induced histamine release from tissues of the three species in a similar way

The observations on antigen induced histamine release from rat and guinea pig tissues have led to the suggestion that the release is the result of enzymatic reactions requiring energy from oxidative or glycolytic processes The results of the present investigation indicate that antigen induced histamine release from rabbit skin may be dependent on a similar release mechanism

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Reduction of the Noradrenaline Content of Skeletal Muscle by Sympathetic Stimulation

By

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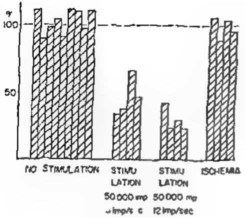
It has been shown that the noradrenaline (NA) content was considerably diminished in several organs of rats subjected to cold stress This diminution did not occur in the presence of a ganglion blocker (Leduc 1961) Moreover a pronounced increase has been seen of the tissue NA content after preganglionic nerve section (Sedvall 1964) and following ganglion blocking substances (Karki Paasonen and Vanhakartano 1959) or bretylium (Ryd 1962) The above results might indicate that the firing rate in sympathetic nerves can affect the NA content of different organs and that the NA stores of some adrenergic nerves are normally slightly reduced by the continuous impulse discharge However even after prolonged nerve stimulation the stores of NA might be found about normal indicating a rapid resynthesis (Luco and Goñi 1948 Euler and Hellner Bjorkman 1955) In this apparently controversial situation it was thought that the question needed further experimental testing

Experiments were performed on cats anesthetized with sodium pentobarbital (30—40 mg/kg) Both sympathetic chains were sectioned at the level of L5 Supramaximal stimuli were given to the right sympathetic chain with bipolar silver electrodes at frequencies of 5 or 12 imp/sec Pulse duration was 5 msec and strength 5—25 V In all the experiments a total of 50 000 impulses were delivered The circulatory effects of the stimulation were in all experiments continuously measured by recording the blood flow through *A femoralis* on both sides using the drop chamber technique described by Lindgren (1958) Blood pressure was recorded from the common carotid artery and was never below 100 mm Hg at the end of an experiment Immediately after the stimulation the cat was killed and *M gastrocnemius medialis* in both legs were dissected out The NA content of the muscles was determined by a modification of the technique described by Haggendal (1963) For each cat the muscle of the unstimulated side served as a control The NA content of muscles the sympathetic supply of which had not been stimulated was on the average 99 ng/g (49—210 ng/g)

The results are summarized in the figure where in each cat the NA content of the right gastrocnemius muscle is expressed as a percentage of the left In control experiments on 8 unstimulated cats the NA content of the muscle on the right side as a percentage of that on the left side was on the average 100 % (90—112 %) In the cats whose sympathetic chain had been stimulated the NA



Noradrenaline content of the right gastrocnemius muscle as a percentage of the left

content was considerably diminished on the stimulated side Stimulation with 12 imp/sec produced an average decrease in the NA content of 70 % (57—76%) whereas with 5 imp/sec the average decrease was 53 % (33—65 %) During stimulation the blood flow on the stimulated side was reduced 2 to 5 times To test that our results were not due to the ischemia caused by vasoconstriction the A *femoralis* of one side was partially occluded for 2 hours in 4 unstimulated cats This procedure did not affect the NA content of the muscles

It has been shown that the NA content of skeletal muscle is predominantly stored in vasoconstrictor nerves (Sedvall 1964 Fuxe and Sedvall 1964) Thus the present results show that in the vasoconstrictor nerves of skeletal muscle the transmitter stores can be considerably diminished by prolonged stimulation even at a low physiological frequency Further investigations are being carried out on the physiology of this decrease in transmitter content

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The Distribution of Adrenergic Nerve Terminals in the Rabbit Oviduct

By

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The motility pattern of the isthmus of the rabbit oviduct differs from that of the ampullary part (Greenwald 1963) Furthermore a preovulatory occlusive mechanism in the isthmus has been described recently (Brundin 1964 a) In this preliminary report morphological evidence is presented which suggests that adrenergic nerve fibers may influence these functional variations in the two parts of the rabbit oviduct

A highly specific fluorescence method for the histochemical demonstration of monoamines including noradrenaline in adrenergic nerve terminals (Falck 1962 Falck Hillarp Thieme and Torp 1962) has been applied to Fallopian tubes from sexually mature rabbits As illustrated in Fig 1 the number of terminals with specific fluorescence increases from the ovarian end of the organ towards the uterus In the infundibulum and ampulla most if not all of the fluorescent terminals are found around blood vessels In the isthmus there is a gradual increase of fluorescent terminals which appear to follow the smooth muscle cells of the circular layer At the tubo uterine junction there is an abundance of nerve terminals in the thick circular musculature (Fig 1 c) Towards the uterus this takes almost the appearance of a sphincter The myometrium however seems to be devoid of fluorescent terminals apart from those seen around the blood vessels (cf Falck 1964)

No terminals were seen among the fat cells in the periisthmic adipose tissue (cf Wirsen 1964)

Parallel biochemical analysis has shown that the noradrenaline content of the isthmus and tubo-uterine junction is up to 5 times higher than that of the infundibulum and ampulla (Brundin 1964 b) The present results suggest that this difference is mainly due to the adrenergic innervation of the gradually increasing circular muscle layer of the isthmus and tubo-uterine junction However recent studies have shown that besides the uterus the smooth musculature of the digestive tract and the ureters (Norberg 1964 a b) are essentially devoid of

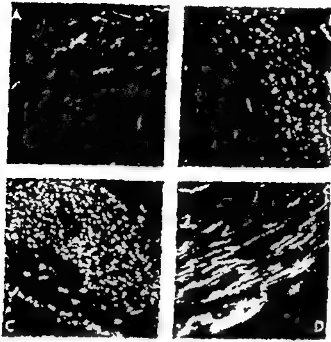

Fig 1 A Infundibulum near the ampulla rabbit Cross-section Only a few fluorescent terminals mainly in the walls of blood vessels 250 ×
B Isthmus Numerous fluorescent terminals in the circular layer of the smooth musculature and around blood vessels The section is slightly oblique 250 ×
C Tubo-uterine junction Abundant innervation of the circular muscle layer The section is slightly oblique 250 ×
D Tubo uterine junction cross section The varicosities of the fluorescent terminals are clearly seen as is the circular orientation 250 ×

direct adrenergic innervation Thus the rich adrenergic innervation of the isthmus and particularly that of the *tubo-uterine junction is noteworthy* It appears likely that this abundant innervation is of importance for the different otility patterns and for the occlusive mechanism and thus also for ovum transport and sperm ascent

A similar histochemical and *biochemical study of the* human Fallopian tube is in progress

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Acta physiol. scand. 1964. 61. 205—211

From the Department of Pathology, Section II, University of Helsinki, and from Central Hospital of Savonlinna, Finland

Effect of Histamine and Insulin on Eosinophils and Mast Cells in the Rat Gastric Mucosa[1]

By

TOIMI RÄSÄNEN

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Abstract

RÄSÄNEN, T. *Effect of histamine and insulin on eosinophils and mast cells in the rat gastric mucosa.* Acta physiol. scand. 1964. *61.* 205—211. — The number of mast cells in the mucosa of the glandular stomach of the rat decreased on account of degranulation 2—4 hours after histamine and insulin injections and after ingestion of food. Re-granulation of the mast cells occurred in a later phase. Morphological changes were established in the mast cell granules during gastric stimulation. Tissue eosinophilia of the lamina propria of the glandular stomach increased at the beginning of the stimulation, decreasing in a later phase. Lethal insulin shock caused an increase in the tissue eosinophilia of the glandular stomach. The role of the heparin and histamine contained in the mucosal mast cell granules during the stimulation of the gastric mucosa, in the phase in which the ion changes and local heparinemia in the mucosal lamina propria perhaps provoke inhibition of eosinolysis, is suggested.

In addition to histamine (Code 1956), sulphurated mucopolysaccharides are secreted in the gastric juice (Smith et al. 1952, Galletti, Inesi and Lodi 1958). Gastric mucosa contains abundant heparin-like polysaccharides (Smith and Gallop 1953) and histamine (Rose and Browne 1940), which are possibly bound with the mucosal mast cells since the decrease in their number in rat gastric mucosa during glucocorticoid effect is accompanied by a decrease in the mucosal histamine content (Foley and Glick 1962). It is possible that the release of endogenous histamine stimulates secretion by the cells of the gastric parenchyma.

Tissue eosinophilia appears to increase postprandially in the mucosa of the gastrointestinal canal (Heidenhain 1888). This has been attributed to the entry of foreign proteins into the organism (Piggart 1932, Godlowski 1953). However, the increase in tissue eosinophilia probably occurs without protein effect under the influence of atropine and vagotomy (Teir et al. 1956), which inhibit gastric

[1] Sponsored by the Sigrid Jusélius Foundation.

secretion Alcohol intoxication causes an increase in tissue eosinophilia in rat gastric mucosa in one hour (Miettinen and Kouvalainen 1958) *Fasting on* the other hand, reduces the tissue eosinophilia of the lamina propria (Heidenhain 1888 Teir et al 1956)

The potassium content rises in the plasma and the juice during gastric secretion (Hollander 1961) As gastric secretion is possibly preceded by rapid changes in the homeostasis of the lamina propria, changes which are probably associated with cellular kinetics, the present investigation was instituted to study the changes in mast cells and tissue eosinophilia during and after histamine and insulin stimulation and during and after a meal

Method

The investigation was divided into 6 exp in which 4-month old male rats of Wistar strain were used The injections and the test meal were given in all the experiments after a 2-day fast during which the rats were allowed water ad lib

The mucosal specimens were taken from the gastric wall immediately aft r decapitation The wall of the glandular stomach which was full of secreta or food contracted rapidly after the opening of the stomach to the size of an empty stomach The preparation of the microscopic samples and the mast cell and tissue eosinophil counts were performed as described earlier (Rasanen 1960 a b) The statistical deviations were calculated by means of Fisher s t test

Histamine Experiments I II and III

Twenty rats were injected intraperitoneally with 10 mg of histamine in the form of dihydrochloride Ten of them were decapitated within 2 and the other 10 within 5 hours of the injection The controls were given 0 5 ml of normal saline 5 hours before decapitation

In experiment II 10 rats (weight 140—160 g) received 3×1 0 *mg* of histamine at min intervals i p and were decapitated 2 hours after the first injections The 10 control rats (weight 135—150 g) were given saline injections

In experiment III 10 rats (weight 110—150 g) received 4×1 0 mg of histamine at 30 min intervals and were decapitated 3 hours after the first injection The 10 control rats (weight 115—155 g) were injected with 0 5 ml of normal saline

Insulin Experiments IV and V

Nine rats (weight 196—260 g) were injected i m with 20 I U of water soluble insulin and decapitated 1—3 hours later when they were in severe shock and in agony The 10 controls (weight 170—240 g) received 0 5 ml saline injections

In exp V 10 rats (weight 124—164 g) were given 4 I U of water soluble insulin and decapitated 4 hours later The 10 control rats weighed 132—172 g They received no injections

Meal Experiment VI

In the last experiment VI the rats were fed raw ground liver Ten rats (weight 146—204 g) were decapitated *1 hour after the beginning of the meal* another 10 rats (weight 136—168 g) were decapitated after 3 hours a third lot of 10 rats (weight 14?—196 g) were decapitated after 5 hours The rats were allowed food throughout the experiment The controls were the same as in the preceding experiment and were decapitated in a fasting state

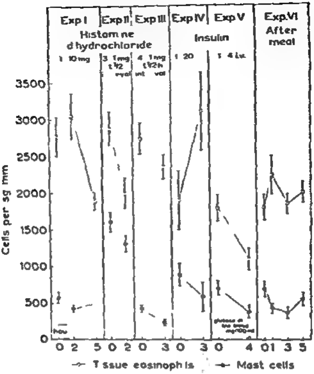

Fig 1 Number of cells per sq mm of tissue ± SE in rat gastric body mucosa after histamine and insulin and after a meal

Results

The number of mast cells and tissue eosinophils per sq mm of tissue ± SE in the body part of the gastric mucosa in all the experiments is to be seen in Fig 1

Mast cells

The superficial profuse mast cell layer of the gastric body mucosa (Fig 2) seemed to become degranulated within 2—3 hours of the first histamine injection The difference was probably significant statistically ($P < 0.05$—0.02) in exp II and III in which the rats received repeated histamine doses The mucosal mast cells were also degranulated ($P < 0.05$) under the influence of a large insulin dose which led rapidly to severe shock In this experiment the mucosal mast cells had disappeared completely in 3 cases from the areas in which some of the superficial and intact part of the mucosa was seen microscopically (Fig 3 A) On the other hand nothing indicative of degranulation was seen in the mast cells of the submucosa in the same test animals (Fig 3 B) Owing to the extensive destruction of the mucosa the zero values of the mast cells of

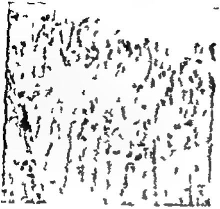

Fig 2 Numerous mucosal mast cells in the superficial part of body mucosa of rat (× 150)

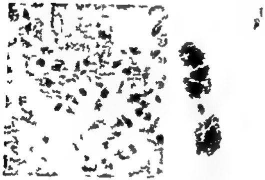

Fig 3 Left Total loss of granulated mucosal mast cells in rat gastri mucosa after severe insulin shock (325) Right No sign of degranulation in the submucosal connective tissue mast cells after insul n shock (600)

Fig 4 Flow of metachromatic material from the mucosal mast cells after insulin injection (600

these 3 rats were excluded in calculating the mean in Fig 1 which is thus 397 cells per sq mm of tissue

A smaller dose of insulin caused degranulation of mucosal mast cells ($P <$ 0 05) in 4 hours The profuse mucosal mast cell zone showed a distinct morphological change *during the insulin shock (Fig 4)* with vacuolisation of the mast cell granules Moreover their metachromatic material spread into the

environment lost some of its metachromatic colour and flowed into the gastric glands in the area of the mucous neck cells

The number of mucosal mast cells decreased ($P < 0.05$) within [illegible] hours of the meal but then began to increase in number in the same way as after a single histamine injection

Tissue eosinophilia

A large histamine dose first produced an increase in tissue eosinophilia and then in 5 hours a fall to below normal ($P < 0.02$) Small repeated histamine injections caused a decrease in tissue eosinophilia in 2—3 hours It was almost significant in experiment II ($P < 0.05$)

A large insulin dose caused an increase in tissue eosinophilia within 1—3 hours during shock Tissue eosinopenia ($P < 0.05$) occurred 4 hours after a small dose of insulin The blood sugar fell from 95 $\pm$ 3.3 mg per 100 ml to 19.0 $\pm$ 2.0 mg per 100 ml

Tissue eosinophilia increased slightly in the first postprandial hour but later returned to the level of the controls

Discussion

Mucosal mast cell degranulation seems to occur in the initial phase of gastric stimulation In this process histamine is probably released from the mast cells and its amount in rat gastric mucosa decreases in connection with the degranulation of the mast cells (Foley and Glick 1962) This endogenous histamine probably functions as a direct stimulant of the gastric parenchyma Mucopolysaccharides appear in the gastric juice and increase during the stimulation (Kowalewski and Silbermann 1958 Galletti Inesi and Lodi 1958) It seems microscopically that the metachromatic material of the gastric mast cells flows into the glandular tubes near the mucous neck cells Observations indicative of this have been made earlier (Grossberg Komarov and Shay 1950)

The mast cell degranulation phase appears to last 2—3 hours after a single histamine injection after that regranulation occurs in the same way as after a meal Repeated histamine injections and especially insulin shock result in a more lasting degranulating process probably by mobilising the pituitary adrenal system and the glucocorticoid component stimulating gastric secretion slowly becomes visible (French et al 1953) After histamine and insulin injections rapid gastric stimulation is probably caused by adrenalin and acetylcholine which most likely function as histamine liberators in the gastric mucosa degranulating its mast cells

The increase in tissue eosinophilia in the initial phase of secretion can hardly be due as has been assumed to the effect of foreign proteins No increase in tissue eosinophilia was encountered in the present work in the later secretory phase when proteins are probably being resorbed Repeated histamine injec

tions caused tissue eosinopenia in 2—3 hours and 4 I U of insulin had the same effect in 4 hours Severe insulin shock on the other hand increases tissue eosinophilia in rats in which the superficial mucosa was partly destroyed and a part of the mast cell layer disappeared into the gastric lumen Probably associated with this process is a local flow of intracellular potassium into the environment Increase in tissue eosinophilia appears to be produced by toxic quantities of alcohol (Miettinen and Kouvalainen 1958) atropine and pilocarpine, and operation stress (Teir et al 1956)

During shock induced by repeated histamine injections the tissue eosinophilia of the gastric mucosa only decreases perhaps as a result of the direct eosinopenic effect of exogenous histamine and released endogenous histamine The potassium content of the plasma and probably also of the lamina propria rises at the beginning of gastric stimulation It also increases in the secreta during stimulation (Harris and Edelman 1960) This initial rise in the potassium content on the nutritive side may provoke an antieosinopenic effect This was suggested by Aschkenasy (1959) for blood eosinophilia

The initial rise in tissue eosinophilia during glucocorticoid effect (Wegelius and Teir 1958) occurs more slowly than the increase induced in the present work by histamine insulin or a meal and secretion is also stimulated more rapidly than under the influence of glucocorticoids (French et al 1953) Mucosal mast cells release histamine during glucocorticoid effect (Foley and Glick 1962) and this histamine reduces in vitro the potential differences in the gastric mucosa and aids the passage of potassium via the mucosa (Harris and Edelman 1960) It is impossible to say yet whether the heparin of the mucosal mast cells which appears to promote the passage of potassium via the renal membranes (Hardegg and Pfleiderer 1959) also participates in this mucosal transport of potassium An initial local heparinemia in the gastric secretory phase may inhibit also eosinolysis as has been observed in systemic blood (Braunsteiner Potuzhek and Thumb 1959)

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Acta physiol scand. 1964. 61 212—218

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An Attempt to Measure the Two-Way Permeability in the Blood-Brain-CSF System

By

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Abstract

EDSTROM R *An attempt to measure the two-way permeability in the blood brain CSF system* Acta physiol scand 1964 *61* 212—218 — *Perfusion of various* CSF (cerebrospinal fluid) spaces with an artificial fluid opens the way to a quantitative *analysis of transport rates and permeability in the blood CSF interphase* Several approaches have been reported recently two of the most successful ones are the reports of Heisey *et al* (1962) and Pollay and Davson (1963) In these studies the CSF blood transfer has been calculated from measured losses of indicator material from the perfusion fluid The present paper will report on an attempt to amplify this approach by a determination also of the appearance rate of the indicator material in the blood and by a simultaneous measurement of the transport rate in the opposite direction blood CSF

Various equations for use in this system are given and practical experiments *with sodium and iodide transfer are reported. They do not permit a conclusion* of any preferential direction of sodium transport but iodide seems to be transported about *10 times faster out of the cerebral ventricles than in the opposite* direction

Method

Cats weighing 3—4 kg have been anesthetized with 2 ml of 6 mebumal *i p* and with later i v supplements if needed One or both lateral cerebral ventricles have been cannulated for the tracer containing inflow Ringer solution All the perfusate has been collected through a plastic tube at the aqueduct technically *ad modum* Bhattacharya and Feldberg (1957 1958) Feldberg and Fleischhauer (1960) In order to study the two-way transfer the procedure has here been supplemented by an i v tracer infusion

The perfused anterior 3 cerebral ventricles are an obviously defined pool *The other pool represented by the circulating* blood plasma will vary with different indicators Its initial size is the distribution space of the indicator It has been obtained by a tracer dilution calculation after an initial i v injection Subsequent *leakage of tracer* material from this *initial distribution volume in the body is equal* to the constant rate at which tracer material has been added i v in order to keep the plasma concentration constant For sodium the indicator fraction leaving the initial distribution *space* each minute was found to be approximately *1 60*

All cats have been tracheotomized and intubated. The arterial blood pressure and the inflow temperature have been continuously monitored.

The two-way transfer of the tracer pair Na^{24} and Na^{22} has been studied and compared with the transfer of I^{132} and I^{131}. Unfortunately the used Te^{132} columns yielded an I^{132} preparation that was contaminated with I^{131}. Therefore for iodide only oneway fluxes in parallel with sodium will be reported. In the two-way studies (with sodium) it has proven convenient to give the more rapidly decaying tracer of the studied element in the perfusion fluid, while the more long lived tracer of the same element has been given i.v.

In an attempt to alter the sodium and iodide flux rates differentially (i.e. an acceleration of one element and a retardation of the other), three electroshocks have been given 2 min apart in the midst of the 2 hour perfusion period. Trains of 25 cps square waves of 50 volts from a Grass S4B stimulator have been applied with 2 finger tip size electrodes and contact jelly to the exposed parietal bones for 5 sec. After the stimulation tetanus sporadic clonic tongue twitching could be observed for about 2 min in the fully barbiturate anesthetized cats.

To record the catheter positions and the possible presence of any leakage, the perfusion system has finally been rinsed with an 0.5 per cent trypan blue solution for 10 min.

The ventricular perfusion rates were determined by gravity. For this purpose the inflow reservoir has been placed on scales and its gradual loss of weight has been recorded. The fluid was forced out of it into the perfusion tubes with air pumped into the reservoir bottle with a pulsating pressure. The actual perfusion pressure in the ventricles has been controlled by the level of the outer tip of the outflow catheter. The latter has been kept 10—20 mm below the level of the external meatus of the ear. The collected outflow fluid has also been weighed at time intervals. The perfusion rates have been kept at approximately 0.3 ml/min. The rate of fluid production in the anterior 3 ventricles should amount to about 5 per cent of this perfusion rate (Dawson *et al.* 1962). The brain tissue with its closely packed cerebral cells may hardly permit any other considerable volume addition or loss. Thus Fick's law may be applied with moderate error on the obtained data on transmural transport between the perfused ventricles and the blood plasma. Any deviant observation may suggest the presence of other transport mechanisms than diffusion.

A Tracerlab well scintillation detector and Superscaler have been used for the radioassay of fluid samples, blood plasma samples and cerebral tissue specimens.

Calculation of permeability coefficients

The following symbols are used:

c_i, c_o, $\bar{c}$, c_p: tracer concentrations in the inflow and outflow perfusate, in the ventricles and in the plasma in percentage of c_i or c_p depending on the source. $\bar{c}$ is related to c_i and c_o as stated by Pappenheimer et al. (1961) thus $\bar{c} = c_o + 0.37 (c_i - c_o)$.

F: the rate of perfusion in ml/min.

A and a: the interphase areas passed by the indicators in cm^2. (The bigger area A is used in plasmato-ventricular transport. The smaller a in ventriculo-plasmatic transport. When both lateral ventricles are cannulated $a = A$, with unilateral cannulation a is about 2/3 A.)

J: the amount of indicator material passing the brain tissue.

V: indicator distribution volume in the body ("indicator space") in ml.

k: the fraction of material lost from the space (V) in unit time.

Δt: time unit (here 1 minute).

K_{vp}, K_{p}, K_{pv}: the diffusional permeability coefficients for ventriculo-plasmatic and plasmato-ventricular transport in cm/min.

With these symbols the diffusional permeability coefficient of Heisey et al (1962) may be calculated thus

$$k_p \quad a = F\frac{c - c}{c}$$

When the tracer appearance rate in the blood is also taken into account two further factors will have to be considered Dilution in the body s distribution space (V) and losses from this space to other body regions

$$\frac{\Delta I}{\Delta t} = V \quad \frac{\Delta c_p}{\Delta t} + V \quad \frac{\Delta c_p}{\Delta t} \quad k$$

but Fick s law gives

$$\frac{\Delta I}{\Delta t} = I_p \quad a \quad (c - c_p)$$

c_p is very small in comparison with the source in the perfusate (c) and may there be neglected With substitutions then

$$I_p \quad a = \frac{V}{c} \quad \frac{\Delta c_p}{\Delta t} \quad (1 + k)$$

This expression permits a direct comparison with the vp permeability figures obtained *ad modum* Heisey *et al* (1962) Any difference between I_p and k_p may be interpreted in terms of retention of tracer material in the central nervous system

In order to determine the plasmato-ventricular permeability (I_p) a constant concentration is upheld in the blood plasma A steady small concentration will result in the steadily perfused ventricular fluid The rate of appearance of the indicator there is equal to its rate of appearance in the outflow fluid

$$\frac{\Delta I}{\Delta t} = F \quad c$$

Fick s law gives for the pv passage

$$\frac{\Delta I}{\Delta t} = I'_{pv} \quad A \quad (c_p - c)$$

The intraventricular concentration is very small in comparison with that in the blood pla ma therefore with substitutions

$$I_{pv} \quad A = F\frac{c}{c_p}$$

A comparison of k_{pv} on one side I_p and k_p on the other will reveal any preferential transport direction of a given indicator and might suggest other transport modes than simple diffusion

Results

Comparison of I_p and k_p

In Table I cats number 3 4 and 5 stand out because of their high I'_p values The data reflect considerable tracer losses from the intraventricular lumina while the low (or normal) I'_p figures show that only a small amount of the escaped indicator material has reached the blood stream In the mentioned 3 animals there was staining of the catheter tracks with blood and or trypan

Table I Diffusional brain permeability coefficients to sodium (cm/min) × area

Cat	Before and after electroshocktreatment					
	K_{vp}	a	K_{vp}	a	K_p	A
1	0.02	0.01	[illegible]	[illegible]	0.01	0.01
2	0.05	0.04	0.03	0.04	0.03	0.03
3	(0.24	0.22)	0.01	0.04	0.01	0.02
4	(0.30	0.34)	0.03	0.01	0.02	0.03
5	(0.28	0.14)	0.01	0.01	0.02	0.01
6	0.06	0.06	0.01	0.01	0.01	0.01
7	0.02	0.02	0.02	0.02	0.01	0.01
9	0.01	0.01				
10	0.04	0.03	0.02	0.01		
11	0.02	0.02		0.02		
12					0.01	0.01
13					0.01	0.02

Bilateral perfusion (a = A) in other experiments a approx = 2/3 A

Table II Diffusional brain permeability coefficients to iodide (cm/min) × area

Cat	Before and after electroshocktreatment					
	K_{vp}	a	K_{vp}	a	K_p	A
9	0.02	0.04	0.04			
10	0.04		0.11			
11	0.06	0.04	0.09	0.10		
12					0.003	0.004
13					0.003	0.006

a approx = 2/3 A

blue after the final rinsing of the perfusion system with this dye suggesting a retrograde leakage of the perfusate along the ventricular catheters into the outer CSF

Apart from these artefactual results a comparison of K_{vp} and K_{vp} shows little or no difference between the ventricular loss and the plasma appearance of sodium Thus there is no evidence (from these figures) of any sodium retention in the brain tissue during its transport in the ventriculo-plasmatic direction The limited figures for iodide favour a similar conclusion for that tracer

The validity of this conclusion has been checked with the data on the final tracer content of the brain tissue The mean radiosodium concentration in the hemispherical brain tissue was only $2.7 \pm 0.4\,\%$ ($\pm$ SEM) of the radiosodium concentration in the original perfusion fluid The concentration in the exposed

choroid plexus tissue was 7 0 ± 1 0% The unknown concentration in the cerebral interphase during the ventriculo plasmatic passage of radiosodium seems to have been effectively removed by the brief ventricular rinsing The tracer concentration in the final outflow fluid is of similar magnitude as in the mentioned tissues the plasma concentration is about 20 times lower These figures support the permeability data that there is practically no retention of radiosodium in the brain tissue during ventriculo-plasmatic transport Similar figures for iodide are (surprisingly) even lower than the sodium figures in the hemispheres (1 ■ %) but are higher in the choroid plexus tissue (31 %)

Comparison of vp and pv transport

The data in Table I fail to give any support for a preferential transport direction of sodium For iodide Table II shows a 10 fold difference between K'_{pv} on one side and K_p and K'_p on the other The data are few, but obtained in experiments that have given fair simultaneous sodium data (Table I)

Control experiments with intravenous trypan blue, where the (tracheotomized) cats were subjected to similar cerebral traumatization and ECT, did not reveal any barrier damage i e undue extravasation of trypan blue in the brain The catheter tracks seem to heal up between the preparation and the start of the perfusion, if this interval exceeds approximately half an hour

A comparison of the permeabilities to sodium och iodide in the vp and pv directions (Table I and II) shows that the ventriculo-plasmatic transport of iodide is about 3 times more rapid than that of sodium, while the plasmato-... lar permeability is about 3 times greater for sodium than for iodide ... these conditions

The effect of electroshock treatment

ECT had no significant effect on the permeability of the brain tissue to sodium or iodide neither in the vp nor in the pv direction, under these conditions as can be seen in Table I and II

Ventricular volume and absolute permeability figures

An analysis of the unilaterally perfused ventricular distribution space of the indicator *ad modum* Heisey *et al* (1962) gave in the cats a figure of 1 1 ± 0 1 ml The ventricular surface areas a och A may thereby be roughly estimated For A the figure would be about 10 cm² which would give a rough estimate for the absolute value of the cerebral diffusion permeability to sodium in the order of 10^{-5} cm/sec in these experiments

Discussion

Direct simultaneous quantitative two-way studies of matter transfer between blood and CSF have to my knowledge not been systematically performed They might be achieved with moderate modifications of some of the published

cerebral perfusion techniques e g Pappenheimer *et al* (1962) or Pollay and Davson (1963) The present approach carries an extra advantage since it isolates the transcerebral passage thus eliminating the obscuring effects of volume flow transport through the arachnoid villi This advantage is gained at the risk of some of the quantitative precision (Davson *et al* 1962) On the other hand an added degree of confidence in the data has been gained here in comparison with the first mentioned two methods since not only disappearance rates from the intraventricular perfusate have been determined but also the appearance rates on the blood side The suggested method for measurement of the size of and the rate of leakage from, the pool represented by the blood plasma will also eliminate problems caused by different plasma-cell distribution in the blood of different indicators

The perfusion technique used here has found some application in qualitative studies (e g Feldberg and Fleischhauer 1960 Klatzo *et al* 1964) and in semi quantitative estimations of blood CSF (e g Domer 1962) and CSF blood transfer (e g Drakoci *et al* 1961) The present study shows that it is possible to obtain significant quantitative data with this technique even to find an absolute figure for the cerebral permeability The reported 10-fold difference between the rapid ventriculo-plasmatic and slow plasmato-ventricular transport for iodide is in agreement with previous expectations (Davson *et al* 1962) The figure is of the same magnitude as that given by Pollay and Davson (1963) for the blood CSF transfer of para amino-hippurate in rabbits

The literature contains several statements about the influence of ECT various psychotropic drugs and mental disease states on the transcerebral transport mechanisms for a review see Edstrom (1964) The validity of such statements can be experimentally tested with the presented method as has been illustrated here with ECT A systematic similar trial of metabolic inhibitors and psychotropic drugs might bring information on the basic transport mechanisms involved and on their possible clinical significance

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Acta physiol scand 1964 61 219—227

From the Department of Physiology Karolinska Institutet Stockholm Sweden

An Occlusive Mechanism in the Fallopian Tube of the Rabbit

By

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Abstract

Brundin J *An occlusive mechanism in the Fallopian tube of the rabbit* Acta physiol scand 1964 *61* 219—227 — The study was undertaken to determine whether a preovulatory occlusive mechanism in the isthmus of the rabbit Fallopian tube separates the cavity of the ampule from that of the uterus as is the case after ovulation An in vitro method for the recording of the intraluminal pressure variations of the isolated rabbit Fallopian tube has been used and is described Evidence for the existence of an active occlusive mechanism in the isthmus of the estrous rabbit Fallopian tube is presented and discussed There is a difference in the sensitivity of the Fallopian tube and the uterine horn to noradrenaline and acetylcholine The ampullary region is more sensitive to acetylcholine than is the uterine horn which is more sensitive to noradrenaline than the ampulla

Earlier works (Burdick and Pincus 1935) have revealed that following ovulation in the mouse or rabbit the eggs were retained in the ampulla of the Fallopian tube for a comparatively long time before continuing down to the uterus This observation led them to postulate that a „tube locking mechanism" might be responsible for the retention of ova in the ampulla Repeated attempts to demonstrate the „tube locking mechanism" by histological techniques have failed (Greenwald 1961) However the functional existence of a postovulatory "tube locking mechanism" has been verified by several studies on rat mouse and rabbit (Burdick Whitney and Pincus 1936 Pincus and Kirsch 1936 Whitney and Burdick 1936 Alden 1942 a b Burdick Whitney and Emerson 1942 Black and Asdell 1958 1959 Harper et al 1960 Greenwald 1961 Harper 1961) It appears as if the passage of ova through the Fallopian tube had not been studied using physiological techniques The aim of the present investigation was to attempt to demonstrate the existence or the absence of a preovulatory functional occlusion in the Fallopian tube of the rabbit

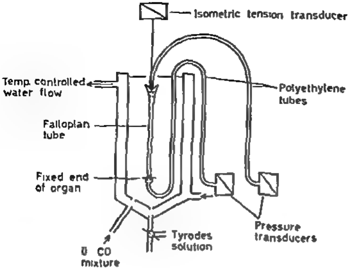

Fig 1 The experimental apparatus

The spontaneous movements of the Fallopian tubes in the intact rabbit include peristaltic waves (Blundell 1819 Westman 1926 Kok 1929 Black and Asdell 1958 1959 Harper 1961) travelling from the infundibulum or the ampulla towards the uterus These spontaneous peristaltic waves should induce variations in the intraluminal pressure (Meschan and Quigley 1938) of the ampulla A failure of these waves to reach the uterine cavity would indicate the presence of an obstacle in the isthmus

In the present investigation intraluminal pressure recordings were made in vitro in the ampulla of the Fallopian tube and in the uterine cavity close to the tubo uterine junction Virgin estrous rabbits were used throughout the study Employing this technique it has been possible to demonstrate a preovulatory tube locking mechanism in the isthmus of the estrous rabbits studied

Materials and methods

Virgin albino rabbits (Swedish Land race) weighing 2 5 – 3 0 kg were used The animals were given food and water ad libitum The usual pretreatment by oophorectomy in this type of studies was attempted earlier but always led to postoperative inflammatory reactions in the fimbrial end of the Fallopian tube Therefore oophorectomy was not employed in this study Instead artificial estrus was induced by daily i m injections of 50 μg estradiol 17 β for ■ days prior to the day of the experiment The pretreatment secured a certain uniformity of the material since it produced estrogenic predominance In addition the estrogen caused an enlargement of the organs which facilitated the experimental procedure

After sacrificing the animals by a blow on the head the Fallopian tubes were dissected out along with 3 – 4 mm of the uterine horn on the respective side The ovaries were inspected during this operation and no signs of ovulation could ever be detected The preparate consisting of the whole Fallopian tube and the transected uterine horn was

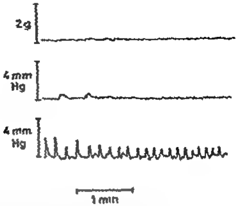

Fig 2 Spontaneous activity of the preparation In this as subsequent records the top tracing represents longitudinal tension middle tracing intraluminal pressure of the ovarian end of the uterine horn bottom tracing intraluminal pressure of the ampulla.

thoroughly cleaned from the surrounding tissues The preparate was then suspended as shown in Fig 1 in a thermostatically controlled organ bath (39 5 ± 0 5 C) The bath contained 75 ml of exchangeable Tyrode s solution of the following composition NaCl 8 0 g KCl 0 2 g $CaCl_2$ 0 2 g $MgCl_2$ 6 H_2O 0 2 g $NaHCO_3$ 1 0 g NaH_2PO_4 H_2O 0 05 g glycose 1 0 g distilled water to 1 000 ml The solution of the bath was aerated by a mixture of O_2 (93 5) and CO_2 (6 5) The isometric longitudinal tension of the preparation was recorded by means of a strain gauge transducer (Grass F 303) The bottom end of the preparation was fixed in the bath The ampullary intraluminal pressure was recorded by the open end of a polyethylene tube filled with Tyrode s solution and inserted 3—4 cm through the fimbrial end of the Fallopian tube The intraluminal pressure in the uterus was recorded by means of a polyethylene cannula of the same type as the former one and attached 1—2 mm below the tubo-uterine junction The other ends of the two polyethylene tubes were connected to a pair of equally sensitive pressure transducers (Statham P23BC) forming a closed system with the cavity of the preparation The transducers were calibrated following equilibration of the system to the different pressures in the bath Measurements of pressure were recorded on a polygraph (Grass 5P1) The spontaneous activity and the induced variations in tension and intraluminal pressures following various doses of acetylcholine and noradrenaline were studied

Results

Spontaneous activity

A typical pressure recording employing the experimental set up described above is shown in Fig 2 The spontaneous longitudinal tension exhibited by this preparation did not vary considerably in any experiment of this series This was also generally true for the intraluminal pressure recorded by the cannula on the uterine side of the tubo-uterine junction However in a few preparations uterine pressure variations were recorded. These appeared as single wave like increases and decreases of the intraluminal pressure The total duration of these waves was 7—20 sec Their amplitude varied from 0 5—4 0 mm Hg

On the other hand the ampullary intraluminal pressure showed continuous spontaneous wave like pressure variations in all of the preparations studied

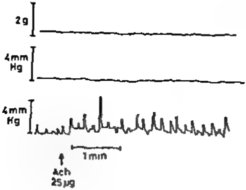

Fig 3 Effect of added acetylcholine Bath concentration 0.3 µg acetylcholine/ml This shows stimulation of the tone and amplitude of the ampullary pressure waves but no effect on the longitudinal tension or the uterine pressure

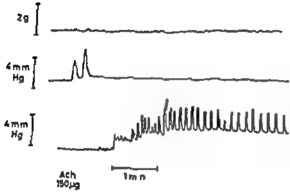

Fig 4 Effect of added acetylcholine Bath concentration 2.0 µg acetylcholine/ml Increased fluctuations of the ampullary pressure waves A rise in the ampullary pressure tone without a reciprocal pressure increase in the uterine lumen indicating isthmic occlusion

The frequency varied considerably from 5—18 waves per minute Their durations was shorter than those of the uterine waves and varied from 2—4 sec In addition the amplitude of the ampullary pressure waves ranging from 0.2—2.1 mm Hg differed from those recorded from the uterine part of the preparation The spontaneous pressure waves in the ampullary cavity were often preceded by a short and slight decrease of the pressure

The intraluminal pressures recorded in the ampullary and uterine ends thus differed concerning frequency duration and amplitude A reciprocal relationship did not exist between the pressures recorded at either end of the tube The same responses were observed regardless of whether the uterine or the fimbrial end was hung uppermost in the bath

Drug induced activity

The experimental set up was tested by the addition of various doses of either acetylcholine or noradrenaline to the bath in an attempt to induce changes in the longitudinal tension and the intraluminal pressures These drugs not only induced variations of the longitudinal tension and of the *intraluminal* pressure

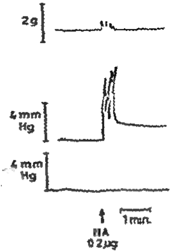

Fig. 5. Effect of added noradrenaline. Bath concentration 0.003 µg noradrenaline/ml. Stimulation of the longitudinal tension and the uterine pressure but no reciprocal pressure increase in the ampulla indicating isthmic occlusion

in the uterine part of the preparation but also influenced the spontaneous activity of the ampulla.

A stimulating effect of acetylcholine on the frequency, tone and amplitude of the ampullary pressure variations occurred at a concentration of 0.3 µg/ml in the bath. Concentrations of 1.3—5.0 µg/ml of bath fluid were necessary in order to induce variations of the uterine intraluminal pressure. Variations of the longitudinal tension did not appear until the concentration amounted to more than 5 µg of acetylcholine/ml in the bath. The responses obtained after the addition of acetylcholine were inconstant throughout the whole concentration range used which was 0.1—8.0 µg/ml bath fluid. Typical responses of two different concentrations of acetylcholine are shown in Fig. 3 and Fig. 4. In spite of the definite increase of the basic pressure in the ampullary cavity, as shown in Fig. 4, no influence on the uterine intraluminal pressure could be recorded. This was a constant observation in all the preparations studied.

In the noradrenaline experiments the concentration used ranged from 0.001—0.13 µg/ml of the bath. The reactivity to noradrenaline was the reverse of that seen after acetylcholine. A concentration of 3 ng/ml of noradrenaline in the bath induced and increased the frequency, tone and amplitude of the rhythmic variations of the longitudinal tension of the preparation that remained relatively constant during the recordings of spontaneous activity. This dose of noradrenaline also produced marked wave-like variations in the intraluminal pressure of the uterine part (Fig. 5). The same dose of noradrenaline not only failed to cause any variations in the ampullary intraluminal pressure, but in addition no increase in ampullary pressure resulted from the enhanced uterine pressure. No effect of added noradrenaline was recorded in the ampulla until the concentration in the bath amounted to 10—13 ng/ml. The effect of this dose is

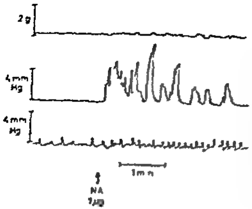

Fig 6 Effect of added noradrenaline Bath concentration 0 13 μg noradrenaline/ml Slight increase of the longitudinal tension variations Induced pressure peaks in uterine lumen but no increase of the pressure in the ampulla indicating isthmic occlusion Slight stimulation of the frequency of the ampullary pressure waves

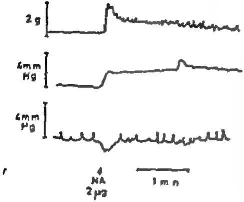

Fig 7 Effect of added noradrenaline Bath concentration 0 3 μg noradrenalin /ml. Induction of longitudinal rhythmic tension variations of increase of the uterine pressure and a simultaneous decrease of the intraampullary tone frequency and amplitude indicate isthmic occlusion

illustrated in Fig 6 where a slight increase of the frequency of the ampullary waves can be noted No intraluminal transference of the uterine cavity pressure was obtained in spite of the great enhancement of the pressure in the uterine cannula In 3 expts out of 13 in the effective dose range studied noradrenaline caused a transient decrease in the tone frequency and amplitude of the intra ampullary pressure waves as shown in Fig 7 However an increase of the tone frequency and amplitude was obtained in 8 out of 13 expts in the effective concentration range No variation of the tone frequency or amplitude was recorded in the two remaining experiments

The induced intraluminal pressure waves recorded from the uterine end of the preparations were generally followed by an enhancement of the intrauterine pressure that could persist for several minutes after the passage of the pressure peaks (cf Fig 5 and Fig 7) The greatest amplitude and the longest duration of the pressure waves were recorded from the uterine end of the preparation and appeared after the administration of noradrenaline An amplitude of 20 mm Hg and a duration of 30 sec were often obtained In spite of the relatively high pre-

sures in the uterine end of the lumen of the preparate no increase in the intra ampullary pressures was recorded in any experiment In this end of the organ the maximal pressure waves were recorded following the addition of acetylcholine However their amplitudes never exceeded 6 mm Hg and the longest duration recorded was 7 sec The ampullary pressure waves often exhibited a short decrease preceding the increasing sloop of the pressure waves even following the addition of acetylcholine or noradrenaline This pattern of the pressure waves was never observed in the uterine end of the preparation

The variability of the responses to acetylcholine was also noted for the noradrenaline induced activities The position of the organ in the bath — uterine or fimbrial end uppermost — was not found to influence the drug induced activities

Discussion

It appears possible to obtain certain information about the individual activities of the longitudinal and circular muscles of the Fallopian tube employing the method described above An undetectable participation of the longitudinal muscles in the production of the pressure waves cannot be excluded However judging from the results in general and from the figures presented the circular muscles would be responsible for the pressure waves recorded except after the addition of noradrenaline that usually stimulated even the longitudinal tension variations

Quantitative assays of the effect of different drugs by the method described have certain disadvantages but the method appears to be valuable for qualitative purposes The disadvantages concerning quantitative recordings is evident by the variability of the responses obtained after administration of the drugs used in the present study This variability could be partly due to different degrees of initial distension of the preparation after equilibration of the intraluminal pressures and to the relatively large volume of the bath necessary for the preparation

The different responses to acetylcholine and noradrenaline were not expected However similar pharmacological differences have been previously described in various parts of smooth muscle organs and also in the oviduct In the guinea pig ileum for instance Munro (1951) reported that adrenaline contracts the ileocoecal region while it relaxes the rest of the small intestine From studies of the longitudinal tension variations of the oviduct of the domestic fowl McKenney Essex and Mann (1932) reported that adrenaline failed to stimulate the isthmus but caused contractions of the other parts of the organ It is also known that certain parts of the human Fallopian tube react unequally to added prostaglandins (Sandberg et al 1962) The discrepancy between the amplitudes recorded in the uterine cannula from those of the ampullary cavity is probably due to the thicker circular musculature in the isthmic part as compared to that of the ampulla (Greenwald 1961)

Presumably, the spontaneous pressure variation recorded in the ampullary cavity during the present experiments are the results of the peristaltic waves travelling from the infundibulum towards the uterus Various reasons exist for such an interpretation of the intraampullary pressure variations Meschan and Quigley (1938) reported that when a peristaltic wave passes the open end of a pressure recording cannula in the cavity of the small intestine the increasing phase of the pressure wave is often preceded by a slight decrease of the resting pressure However this is only an occasionally occurring phenomenon during recordings of peristalsis (Brody et al 1950) In the present experiments a similar decrease of the ampullary pressure often preceded the real pressure wave, both during spontaneous and drug induced activity It should be noted that such a phenomenon has never been recorded from the uterine lumen in the present series of experiments This could indicate that the pressure enhancements of that end were due to mass contractions of the circular muscles and not a result of the propagation of the peristaltic waves from the Fallopian tube

Whatever the origin of the recorded pressure variations in the ampulla may be, they were not observable in the uterine end of the preparation during the recordings of spontaneous activity

In spite of extensive acetylcholine stimulation resulting in an increase of the basic ampullary cavity pressure no signs of enhanced intrauterine pressure were recordable (cf Fig 4) Anatomically the communication between the ampulla and the uterine horn is an established fact in the healthy Fallopian tube It is also evident that an intrauterine pressure of as much as 20 mm Hg uced by added noradrenaline never was transferred to the ampullary cavity view of these considerations some kind of functional occlusion must have present in the isthmus of the Fallopian tubes studied If the present results are transferable to the living intact animal they would imply that even before ovulation a tube locking mechanism in the isthmus of the Fallopian tubes of the estrous rabbit separates the ampullary cavity from the lumen of the uterine horn

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Acta physiol scand 1964 61 228—237

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Further Studies on the Effect of Radio Frequency Hypothalamic Lesions on Lactation and Water Metabolism

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Abstract

GALE C C *Further studies on the effect of radio frequency hypothalamic lesions on lactation and water metabolism* Acta physiol scand 1964 *61* 228—237 — In two lactating goats production of median eminence lesions by radio frequency heating blocked milk synthesis and evoked diabetes insipidus (D I) After administration of hormonal therapy (ACTH STH T 3 and insulin) had restored milk production withdrawal of STH from the regimen was associated with a decline in milk yield The acute polyuria elicited by median eminence lesions in two unanesthetized goats preceded by several hours an increase in water intake (polydipsia) Similarly the onset of the permanent phase of D I in one goat studied was occasioned by a primary polyuria Intravenous injection of a blocking dose of thyroxine two hours before lesioning of the median eminence failed to prevent the thyroid activation which acutely follows such hypothalamic injury

Recent studies have shown that the destruction of the basal hypothalamus in goats either by proton irradiation (GALE and LARSSON *1963*) or by *radio* frequency (RF) heating (GALE 1963) markedly depresses milk synthesis and produces diabetes insipidus (D I) This impairment in milk production was attributed to deficiencies in secretion of anterior pituitary hormones other *than* prolactin since replacement therapy with ACTH somatotrophin (STH) tri iodothyronine (T 3) and insulin restored milk yield As little is known *about* the role of the central nervous system in regulating the secretion of STH one purpose of this study was to investigate further the importance of *this hormone* in repairing lactational defects in goats with brain lesions

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A second object was to distinguish the primacy of polyuria or polydipsia in experimental D I by use of RF lesions in unanesthetized goats

Increased thyroid activity, as shown by rising levels of plasma protein bound iodine (PBI) has been observed to occur acutely after injury to the median eminence (Andersson *et al* 1963) Since administration of exogenous thyroxine suppresses the thyroid activation which normally follows local brain cooling (Andersson Gale and Ohga 1963) a third purpose of this study was to investigate the ability of thyroxine to block lesion induced thyroid activity

Methods

Care of the animals measurements of lactation of water intake and of urine output and electrolyte concentration

The general care of the three goats (two lactating "N D and R and one non lactating "M) and the methods for measuring lactation drinking pattern and urine volume and concentration of Na^+ and Cl^- have been previously described (Gale 1963) In addition on the day of lesioning the pre lesion and post lesion urine was caught *in toto* directly into beakers at micturation. In goat "M urine voided during the onset of the permanent phase of DI was collected in the same manner Urine excreted o er night was collected under oil for the first few days after lesions

Production of RF lesions in the hypothalamus

The technique for implanting needle electrodes permanently in the hypothalamus and later of producing RF lesions in the unanesthetized animal has been reported (Gale 1963) Two pairs of electrodes were implanted in goats N D and M and one pair in goat R Since the 5-minute heating required to produce brain lesions also evoked an acute urge to drink in the goats it was necessary to restrain them from the water buck ts at this time When heat loss reactions (panting and vasodilatation) had subsided within several minutes of stopping RF warming the animals were permitted free access to water

Replacement therapy

Varied combinations of the following hormones were injected subcutaneously into the neck region of two goats, N D and "R (daily dosage) somatotrophin (STH) NIH GH S-5 ovine[1] 12 mg ACTH Schering Corporation ACTH Depot 3 I U tri iodothyronine (T 3) Smith Kline and French[2] 0 7 mg and protomine zinc insulin Vitrum 8 I U

Thyroxine Roche 2 mg was injected into the jugular vein in two goats N D and R two hours prior to median eminence lesions The method for measuring thyroid activity (changes in levels of PBI^{131}) was that used in previous studies (Andersson *et al* 1963) Thyrotrophin (TSH) Ferring Actyron" 0 5 I U was injected into the jugular vein several days after lesions

Histology

The goats were killed by decapitation under nembutal anesthesia The brains were fixed and embedded in celloidin as described previously (Gale 1963) and were cut transversely in 30 μ sections Every tenth section was stained with toluidine blue Pituitary

[1] The somatotrophin was a gift of the Endocrine Study Section National Institutes of Health U S P H S

[2] The tri iodothyronine was a gift from the Smith Kline and French Co

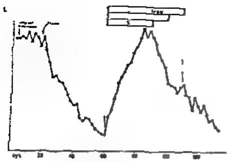


Fig 1 A.

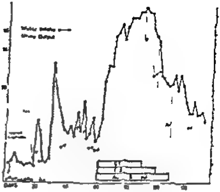


Fig 1 B

Fig 1 A *Block of lactation by median eminence lesions — restoration by hormonal replacement excluding prolactin* In goat "N D" the block of lactation by lesions was corrected by hormonal therapy excluding prolactin suggesting a continuous secretion of this hormone Upon withdrawal of STH from therapy milk production fell from 105 to 60 of pre lesion levels Withdrawal of T 3 and ACTH and insulin were associated with further decline in milk yield (Note each point represents the average of 2 days) Daily dose STH 12 mg T 3 0 7 mg ACTH 3 I U insulin, 8 I U

Fig 1 B *Experimental D I evoked by median eminence lesions — water turnover augmented by hormonal replacement* The tri phasic syndrome of experimental D I in goat "N D" is shown (Note each point represents the average of 2 days) The subsidence of water turnover during the permanent phase (days 30—40) is paralleled by a fall in milk yield (Fig 1 A) Hormonal replacement which elevated D I to a maximum (day 90) also restored milk production to pre lesion levels. Permanent D I was produced in this animal

glands were embedded in paraffin and cut transversely at 10 μ Representative sections throughout the gland were stained with hematoxylin and eosin Thyroid adrenal and ovarian glands were weighed fixed embedded in paraffin cut at 10 μ and stained with hematoxylin and eosin

Results

1 *Block of lactation by RF hypothalamic lesions — restoration of milk production by hormonal replacement excluding prolactin*

The production of hypothalamic lesions by RF heating in two lactating goats caused milk production to decline within 40 days to 14 % and 35 % respectively of pre lesion control values (Fig 1A and 2) The lesser impairment occurring in goat "R" is believed related to the incomplete ablation of the median eminence Only two electrodes were implanted in this animal's brain in contrast to goat "N D" which bore four electrodes and which displayed at histology complete destruction of the median eminence (Fig 3)

When replacement with ACTH STH T 3 and insulin was instituted in these animals milk yield was restored within 15—20 days to 110 % in "N D" and to 90 % in "R" of pre lesion levels In R however this high degree of restoration could not be maintained milk yield falling somewhat to 80 of control

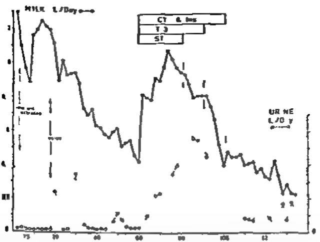


Fig 9 *Effect of hypothalamic lesions in goat R* Lesions blocked milk production and evoked D I Water turnover returned within normal limits after onset of the permanent phase Hormonal therapy restored milk yield and caused a return of D I Daily dose STH 12 mg T 3 0 7 mg ACTH, 3 I U insulin 8 I U

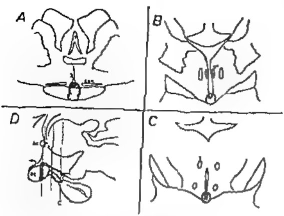
A *Effect on milk production of selective withdrawal of hormones*

When STH was selectively withdrawn from replacement therapy milk yield declined in the following 10 day period to 60 % for "N D and 67 % for "R of pre lesion control This reduction represents the steepest fall in milk yield for "N D i e from 105 % down to 60 % during hormonal withdrawal This was less marked in R from 80 % to 68 % During the 10-day interval after withdrawal of T 3 lactation declined to a greater extent in "R 68 % to 40 % than in N D 60 % to 55 % When ACTH and insulin injections were stopped as well milk yield fell after 10 days from 55 % to 39 % for "N D and from 40 % to 35 % for "R By 25—30 days after cessation of all therapy lactation had declined in both goats to 18 % of pre lesion levels Both animals appeared to be in good health when killed at this time for histology in spite of the condition of D I During the study they had demonstrated good appetite which became pronounced after hypothalamic lesions At sacrifice laparotomy revealed a great quantity of internal fat

II *Diabetes insipidus produced by RF hypothalamic lesions*

A *Rapidity of onset of polyuria*

Lesioning of the median eminence in three unanesthetized goats elicited the classic tri phasic syndrome of experimental D I (Fisher Ingram and Ranson 1935) (Fig 1 B) The most rapid onset of the acute phase of D I occurred in goat "M a non lactating animal which developed a maximal level of polyuria 3 hours post lesions (Fig 4) Onset of polyuria was almost as rapid in "N D urine secretion rate (USR) rising from a control of 0 3 ml/min to 2 0 ml/min



son = supraoptic nucleus
a = anterior lobe of pituitary
n = neural lobe of pituitary
f = column of fornix
p = pineal gland
3 = third ventricle

Fig 3 *Schematic drawing of a hypothalamic lesion causing block of lactation and permanent D I* Cross hatched area depicts the lesion *in* goat N D Atrophy of the neural lobe of the pituitary infarction of the anterior lobe and loss of neurons in the paraventricular and supraoptic nuclei occurred
A B C = Transverse sections through the hypothalamus at the levels *indicated in D*
D = Midline saggital plane of the diencephalon of the goat
ac = anterior commissure
ic = internal capsule
oc = optic chiasm
ot = optic tract
mb = mammillary body
mt = mammillothalamic tract
pvn = paraventricular nucleus

within four hours post lesions associated with a fall in specific gravity from 1 056 to 1 010 In R an elevation in USR from 0 4 ml/min to 1 4 ml/min accompanied by a drop in specific gravity from 1 033 to 1 015 occurred about 12—16 hours after lesions

B *Primacy of polyuria over polydipsia*

Since the primacy of polyuria *vs* polydipsia *in* experimental *D I remains* unsettled (WOLF 1958) the drinking behavior of the animals acutely after lesions was of major interest In goat M it was clearly evident that polyuria preceded polydipsia (Fig 4) Similarly in N D polyuria came before polydipsia In this goat USR reached a maximum of 2 0 ml/min and specific gravity had fallen to 1 010 within 4 hours post lesions but no water was ingested until 6 hours post lesions By this time the goat had excreted 224 ml of urine and then drank 250 ml of water Twenty one hours after lesions the animal had drunk 3 8 l of water and had excreted 2 2 l of urine (specific gravity 1 004) *In* R the acute phase of D I developed during the night some 12—16 hours *post* lesions when no direct observations were made The primacy of polyuria therefore was not verified

C *Normal interphase and onset of the permanent phase of D I*

Since a detailed description of the normal interphase and the onset of the permanent phase of *D I has not previously been reported in the goat these* events were studied in M In this animal the two and one half day period of acute D I was followed by a well-demarcated normal interphase lasting five days and characterized by a very low urine output of *high specific gravity* (1 060)

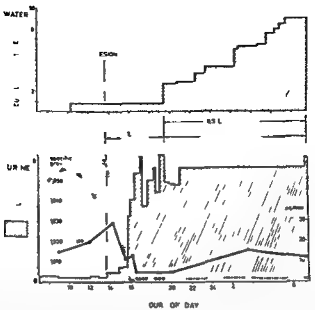


Fig 4 *Primary of polyuria after hypothalamic lesions in an unanaesthetized goat* During the pre lesion control interval goat M usually drank 0 8 l of water each day shortly after eating the morning food ration. On the day of lesioning the goat drank at 10 (upper histogram) Hypothalamic lesions at 14 00 evoked a maximal urine secretion rate within 3 hours (lower histogram) No water however was ingested until 5 1/2 hours after lesions when the goat drank 2 0 l to compensate for the loss of 1 5 l of urine Thereafter polyuria and polydipsia proceeded with a similar time course

With the onset of interphase water intake reverted to the pre lesion pattern of about 0 8 l per day In the second day of interphase however the goat drank no water at all On the morning of the sixth day the animal drank 0 7 l of water as usual after eating its daily food ration By 16 00 however the specific gravity had fallen from 1 065 to 1 026 thus signifying the onset of the permanent phase of D I Between 16 00 and 18 00 USR increased to 1 3 ml/min and specific gravity dropped further to 1 014 At this time — approximately 17 00 — the goat drank 0 5 l of water the first since the morning postprandial ingestion

Thus as in the acute phase of D I it appeared that the secretion of a dilute copious urine had preceded the ingestion of water in amounts above the daily basal need Thereafter polyuria and polydipsia proceeded with a similar time course By the next morning the goat had drunk 2 7 l of water and had voided 2 2 l of urine (specific gravity 1 008) Water turnover did not remain elevated however By the fourth day of the permanent phase water intake urine output and specific gravity were again within normal limits This amelioration of D I is believed due to incomplete ablation of the median eminence

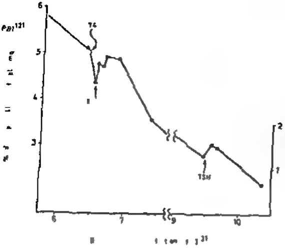


Fig 5 *Failure of thyroxine to suppress activation of the thyroid gland by median eminence lesions* The promptness of blocking action of 2 mg of thyroxine (T 4) injected i v (at the arrow) in goat N D is shown by the sharp downward slope of the PBI[1] curve before lesions The downward slope of PBI[131] prior to thyroxine was based on daily measurements (not shown) after the goat was labelled with I on day 1 The acute rise in PBI [1] after lesions is comparable to that elicited by injection of TSH (0 5 I U) 3 days later Similar results were obtained in goat R

D *Effect of hormonal replacement on D I*

Since in a previous study (Gale 1963) hormonal therapy augmented water turnover in goats with D I attention was paid to the relative importance of hormones in elevating water metabolism in goats N D (Fig 1 B) and R (Fig 2) Apparently, secretion of ACTH, TSH, and STH from the anterior pituitary is necessary for maximal development of D I

III *Activation of the thyroid gland by median eminence lesions*

In goats N D and R lesioning of the median eminence evoked a prompt activation of the thyroid gland as measured by a rise in plasma PBI[131] This response occurred despite administration of a blocking dose of thyroxine two hours before brain lesions (Fig 5)

IV *Hair loss after RF hypothalamic lesions*

Four weeks after brain lesions in R a marked loss of hair occurred Hand fulls of hair could easily be pulled out from the back and shoulders with desquamation of the superficial epidermis This hair loss became most severe before hormonal replacement Several months after lesions and after replacement therapy had been instituted a new coat of hair had begun to grow When the goat was killed no pathological skin condition was apparent A similar loss of hair had been observed earlier in a goat with proton radiolesions in the median eminence (Gale and Larsson 1963)

V *Effect of hypothalamic lesions on electrolyte excretion*

The effect of hypothalamic lesions on urinary excretion of Na and Cl was studied in all three goats In no case was a marked increase in electrolyte excretion noted (Fig 4) This finding is in contrast to the salt wastage which has

been observed to occur in albino rats maintained on a low Na^+ diet and subjected to direct current electrolysis in the hypothalamus (Keeler 1955 Gale and McCann 1960) The discrepancy in results may be due to varying lesion placement or to the different effects of electrolytic *vs* RF heat lesions (Reynolds 1963)

VI *Histology*

A. *Brains*

Destruction of the median eminence was virtually complete in N D (Fig 3) RF heating produced a bilaterally symmetrical lesion which extended from the caudal optic chiasm backwards in the midline through the separation of the pituitary stalk There was a very marked reduction in the number of neurons in the supraoptic nucleus (SON) and in the magnocellular portion of the paraventricular nucleus (PVN) although neither the electrodes nor the heat lesion involved these nuclei directly Despite damage to the caudal optic chiasm no gross impairment in the goat s vision was evident In M an asymmetrical lesion was produced which failed to destroy the caudal median eminence bilaterally Damage to the optic chiasm caused impaired vision Although degeneration of the neurons in the SON and PVN occurred this was less complete than in N D In R the lesion was located in the ventral portion of the middle hypothalamic region but failed to penetrate downward sufficiently to destroy the median eminence totally

B *Pituitary glands*

Pituitary glands of all three goats appeared approximately the same The adenohypophyses were markedly infarcted particularly the anterior portions presumably due to damage to the hypothalamic hypophysial portal vessel system The neural lobes were atrophied No obvious change was discerned in the intermediate lobes

C. *Thyroid glands*

In N D the goat displaying the most complete damage to the median eminence the thyroid gland was smaller (2 0 g *vs* a mean of 3 5 g formalin weight of right lobe) and appeared to contain appreciably less colloid than those of the other goats This is in accord with the known ability of hypothalamic lesions to impair thyroid function (D Angelo and Traum 1956 Andersson *et al* 1963)

Discussion

In the present and in a previous study (Gale 1963) lactating goats with hypothalamic RF lesions produced less milk when STH was withdrawn from a hormonal regimen including ACTH T 3 and insulin These data therefore suggest that impaired secretion of STH is a major cause of the lactation

block which follows disconnection of the anterior pituitary lobe from CNS regulation Since the anterior lobes revealed central necrosis indicative of an interrupted portal vessel supply the role of the CNS in controlling STH secretion during lactation remains in doubt Depression of adenohypophysial function secondary to ischemia could account for a reduced secretion of STH It is indeed striking that lactating goats with hypothalamic radiolesions (produced by a proton beam) showed less severe block of lactation absence of infarction in the anterior lobe and a smaller galactopoietic response to STH injections (Gale and Larsson 1963)

The complete restoration of milk production afforded by a therapeutic regimen excluding prolactin suggests that a high level of synthesis and release of this hormone was occurring in the viable parenchyma of anterior lobes deprived of their normal hypothalamic connections This provides additional support for the theory that prolactin secretion is regulated by central nervous inhibitory processes (Gale 1963)

The technique of producing lesions in the brains of unanesthetized goats has provided clear evidence that polyuria precedes polydipsia in experimental D I Since the goats remained in apparent good condition during and following the lesioning procedure and presumably were capable of drinking as soon as they experienced thirst it appears that polydipsia lags several hours behind the secretion of a copious dilute urine Smith and McCann (1962) have reported that electrolytic destruction of the median eminence in acutely nephrectomized rats deprived of food resulted in the ingestion of amounts of water significantly greater than in nephrectomized controls Such primary polydipsia however might be due to irritant effects on a hypothalamic drinking center of metal deposited by direct current electrolysis (Reynolds 1963)

The five minutes of RF heating required to produce brain lesions induced an immediate strong urge to drink in the goats in confirmation of earlier studies demonstrating a thermostatic component in the regulation of water intake (Andersson and Larsson 1961 Andersson Gale and Sundsten 1962) Acute thirst evoked by RF heating is more likely a result of conductive warming of the preoptic heat loss center than to injury to the basal hypothalamus Immediately that heat loss reactions (panting and vasodilatation) had subsided the animals were permitted access to water No water however was ingested until a clear cut polyuria had developed

In all three animals the typical three phases of experimental D I were well demarcated but only one goat maintained D I throughout the three and one half months of observation This animal revealed total destruction of the median eminence atrophy of the neurohypophysial lobe and marked reduction in number of neurons in the PVN and SON The subsidence of D I during the permanent phase in the other animals was apparently due to incomplete destruction of the supraoptico-hypophysial tracts and to recovery of this system from the effects of edema and sub-lethal injury Although the neurohypophysial

lobes were atrophic there was appreciably less depletion of neurons in the PVN and SON It appears that three anterior pituitary hormones — ACTH STH and TSH — are essential for the development of maximal D I in the goat The moderate degree of D I which can be sustained permanently in the absence of these hormones depends upon the completeness of destruction of the supraoptico-hypophysial system

The stimulus of acute injury to the median eminence broke through a thyr oxine blockade and activated the thyroid gland in a manner resembling an in ection of TSH Most likely such hypothalamic injury acts to release pituitary TSH secondary either to damage to the adenohypophysis or to release of a stimulatory neurohumor from the brain (Schreiber *et al* 1963)

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Acta physiol scand 1964 61 238—243

From the Department of Physiology Karolinska Institutet Stockholm Sweden

The Influence of Reserpine on the Intracellular Distribution of Noradrenaline in the Rat Heart

By

G E JOHNSON[1]

Received 26 November 1963

Abstract

JOHNSON G E *The influence of reserpine on the intracellular distribution of noradrenaline in the rat heart* Acta physiol scand 1964 *61* 238—243 — Rats exposed to a cold stress or treated with a low dose of reserpine were killed at various times and their hearts removed and homogenized in phosphate buffer Subsequent centrifugation of the homogenate produced three fractions 1) a coarse fraction following low speed centrifugation 2) a particulate fraction and 3) a supernatant fraction obtained by high speed centrifugation of the first supernatant These 3 fractions plus aliquots of the total homogenates were assayed for noradrenaline content Following reserpine 0 1 mg/kg a preferential fall in the noradrenaline concentration in the supernatant fraction occurred after 1 hour without any fall in the noradrenaline content of the particulate fraction Two hours after reserpine the total noradrenaline content was reduced to about one half with the same relative proportion of amines in the particulate fraction and in the supernatant as after 1 hour Cold stress inducing a strong increase in noradrenaline release failed to alter either the total noradrenaline content of the hearts or the intracellular distribution of this catecholamine It is suggested that the amine fall in the supernatant fraction after reserpine results from an impairment in the release of noradrenaline from the particulate fraction

In recent years a considerable body of evidence has accumulated suggesting that the noradrenaline found within adrenergic nerves and peripheral tissues does not exist in one homogenous pool Euler and Hillarp (1956) reported that the high speed centrifugation of cell free homogenates of bovine splenic nerves and of rat spleen produced a sediment which contained about 20—30 % of the total noradrenaline activity in bound form while the remainder occurred in free form Later isotopic and pharmacological evidence has given further proof

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that the noradrenaline found in peripheral tissues exists in at least two forms (Hillarp 1960 Axelrod Hertting and Patrick 1961 Trendelenburg 1961) Upon homogenizing puppy hearts and subjecting them to high speed centrifugation Wegmann and Kako (1961) demonstrated the presence of particle bound catecholamines in this organ Direct evidence for the existence of more than one pool of noradrenaline has also been presented for the rat heart and salivary glands (Potter and Axelrod 1962) for the dog heart (Campos and Shideman 1962) and for the cat heart (Campos Stitzel and Shideman 1963) From these and other works it has been postulated that noradrenaline exists within the nerve cell in at least two pools commonly referred to as the "bound and free forms

The ability of reserpine to reduce the catecholamine content of various organs has been well demonstrated (Carlsson and Hillarp 1956 Holzbauer and Vogt 1956 Weil Malherbe and Bone 1959 Muscholl 1959 and others) In view of this effect on the whole organ a study was undertaken to determine the effect of reserpine on the intracellular distribution of noradrenaline in the rat heart A low dose of reserpine was selected to insure that the total depletion of noradrenaline would take several hours In this way any intracellular variation could more easily be observed

Exposing an albino rat to a cold stress of 2 C induces a marked increase in the urinary excretion of noradrenaline (Leduc 1961) The inclusion of cold exposed rats in this study enabled a comparison between the effects observed following a large physiological release of noradrenaline and those produced by the pharmacological agent reserpine

Methods and materials

All experiments were carried out on male rats of the Sprague Dawley strain purchased from Anticimex Stockholm Rats were kept at least one week at room temperature (20 C) before being used for experimentation The weights of the rats ranged from 300 to 350 g

Reserpine (Serpasil kindly placed at our disposal by AB Ciba Produkter Vallingby) was administered in a dosage of 0 1 mg/kg i p and the animals were killed one or two hours after treatment Rats treated with reserpine solvent were killed two hours after injection Cold exposed rats were placed at 2 C for 24 hours before being sacrificed

The animals were killed by a blow on the head and the hearts quickly removed blotted dry and weighed Three hearts were pooled for each experiment. The homogenization fluid was made up of 33 ml of 0 13 M potassium phosphate buffer (pH 7 5) plus [illegible] ml of ethylene diamine tetraacetic acid solution (100 mg/ml) Homogenization and centrifugation were carried out at a temperature of 2 C The hearts were first homogenized in 15 ml of the above solution for [illegible]0 sec in an Ultra Turrax Homogenizer The resulting mixture was then ground for 30 min between two carborundum stones one held fast while the other was motor driven Following homogenization of the hearts the liquid was poured off and the homogenizer rinsed with the remaining 20 ml of buffer EDTA solution This rinsing fluid was then added to the original homogenate Ten ml of the homogenate was removed for the estimation of the total catecholamine content and the

Table I Noradrenaline concentrations (ng/g or % of the total content) of the various fractions of rat hearts following homogenization and differential centrifugation Standard errors of the P/S values are also given

Treatment	Number of expts	Total content	Coarse fraction	Particulate fraction	Supernatant fraction	Recovery	P/S
Control Rats	5	548	179 33%	198 36	131 24	508 93	1 51 ± 0.08
Cold exposed Rats	4	566	192 34 %	185 33	126 22 %	503 89%	1 47 ± 0 10
Reserpine Solvent	4	578	210 37	175 30	124 21	509 88%	1 41 ± 0 10
1 hour after reserpine	6	492	178 36	184 37	96 20	498 93%	1 92 ± 0 11
2 hours after reserpine	4	258	92 36	105 41	55 21 %	252 98%	1.91 ± 0 11

remaining mixture centrifuged at 2 000 × g for 5 min to remove the coarse fraction The resulting supernatant was centrifuged at 147 000 × g in a Spinco Model L Ultra centrifuge for 30 min yielding a particulate fraction and a supernatant fraction The noradrenaline from the 4 fractions 1) total homogenate 2) coarse fraction 3) particulate fraction and 4) supernatant fraction was extracted with trichloracetic acid The extracts were subsequently purified by running through alumina columns at pH 8 3 and the noradrenaline estimated fluorimetrically by the method of Euler and Lishajko (1961 a)

Results

The results are presented in Table I The recovery values given expressed both as ng per gram of tissue and the percentage of the total content were obtained by the addition of the coarse fraction the particulate fraction and the supernatant fraction and amounted to 88 to 98 per cent of the total content From Table I it is seen that neither cold exposure nor the reserpine solvent significantly altered the tissue content of noradrenaline or the relative intracellular distribution It should also be noted that the particulate/supernatant ratio (P/S) remained constant in these experiments One hour after reserpine treatment the total content of the hearts showed a small insignificant fall In addition the noradrenaline in the coarse fraction and in the particulate fraction remained at control values However the quantity of noradrenaline in the supernatant fraction fell significantly one hour after reserpine (P – 0 02) The P/S ratio also showed a significant increase from 1 51 to 1 92 (P – 0 01) Two hours after reserpine all values were depressed but the P/S ratio remained at 1 91

Discussion

Euler and Hillarp demonstrated in 1956 that noradrenaline is largely stored in discrete cytoplasmic granules found within the nerve axon and in organ homogenates Following stimulation of an adrenergic nerve the noradrenaline released is assumed to be drawn from a free pool which is subsequently replenished from the granules in response to a fall in the cytoplasmic concentration of the catecholamine (Euler and Lishajko 1962 cf Hillarp 1960)

The total content for control hearts reported here compares well with previous results published from this laboratory using trichloracetic acid directly to extract the noradrenaline (Johnson 1963) It can thus be assumed that no noradrenaline was lost during the homogenization In the present study 3 fractions of noradrenaline have been obtained The noradrenaline found in the supernatant fraction probably represents what has been referred to as "free or available noradrenaline (Trendelenburg 1961) The assumption is also made that the particulate fraction obtained only after high speed centrifugation is analogous to the "bound noradrenaline found in cytoplasmic granules obtained from adrenergic nerves (Euler and Hillarp 1956 Euler 1958) Interpretation of the coarse fraction is most difficult Undoubtedly some part of this fraction will disappear when more refined methods are developed for homogenizing the tissues The coarse fraction may also contain the noradrenaline that is bound to such relatively large structures as cell membranes It was interesting to observe that following reserpine treatment the noradrenaline content of the coarse fraction behaved similarly to that found in the particulate fraction The noradrenaline in both fractions showed no alteration one hour after reserpine but two hours after treatment diminished by approximately 50 % It cannot be discounted at this time that a third noradrenaline pool obtained after low speed centrifugation exists However because of the uncertainty concerning the coarse fraction only the ratio particulate fraction/supernatant fraction (P/S) is given It was hoped that this ratio would indicate the relative intracellular distribution between the bound and "free noradrenaline

In the experiments reported here the administration of reserpine significantly increased the P/S ratio from 1 52 to 1 91 This change first observed 1 hour after treatment indicates a preferential loss of noradrenaline from the supernatant fraction Is was noted that although no significant changes had occurred in the total content coarse fraction or particulate fraction 1 hour after reserpine the concentration of nordadrenaline in the supernatant fraction had dropped significantly Moreover even though 2 hours after reserpine treatment all the fractions showed significantly lower noradrenaline values the P/S ratio remained at 1 92 It has been suggested that the normal sympathetic nervous activity initially releases "free noradrenaline Normally this is assumed to be replaced by noradrenaline released from the "bound or particulate form (Euler and Lishajko 1962) In view of the observation that the P S ratio increased

following reserpine it might be concluded that the administration of a low dose of reserpine impairs the release of noradrenaline from the particulate fraction In agreement with this conclusion it has been shown that the addition of low concentrations of reserpine to a suspension of adrenergic nerve granules inhibits the release of noradrenaline from these granules (Euler and Lishajko 1961 b) Weil Malherbe and Bone (1959) reported that an i v injection of 0 25 to 0 5 mg/kg reserpine caused a more pronounced disappearance of the amine from the soluble cytoplasmic fraction of the rabbit brain tissue than from the particulate fraction

It might be argued that the increase in the P/S ratio could result from a temporary sympathetic stimulation following reserpine This suggestion is not supported by the observation that following cold stress at a time when there is a large increase in the noradrenaline secretion (Leduc 1961) the intracellular distribution of noradrenaline remained unaltered

Green and Sawyer (1960) administered 5 mg/kg of reserpine i p to rats and measured the intracellular distribution of noradrenaline in brain tissue They reported that this dose of reserpine released noradrenaline from the stored reserves into the cytoplasmic fraction within 15 min Thus it appears that although a small dose of reserpine 0 1 mg/kg depresses the release of noradrenaline from the particulate fraction, a 50-fold increase in dosage releases noradrenaline from this pool Similar results have been published by Euler and Lishajko (1960 1961 b) They reported that although small concentrations of reserpine depressed the release rate of noradrenaline from isolated adrenergic nerve granules large amounts of reserpine increased the release This point is further illustrated by the results of Campos and Shideman (1962) They observed that 80 min after the i v administration of 0 5 mg/kg of reserpine to dogs the noradrenaline in the particulate fraction fell while no change occurred in the soluble (supernatant) fraction However the interpretation of their results is rendered more difficult by the accompanying observation that the percentage recovery fell from a control of 97 per cent to 77 per cent 80 min after reserpine

In summary it can be stated that following the administration of a low dose of reserpine to rats a new P/S ratio is established This change from 1 52 to 1 91 is established within the first hour after treatment It has been suggested that this alteration in the intracellular distribution of noradrenaline is due to an impairment in the normal physiological release of noradrenaline from the particulate fraction The mechanism behind this effect remains obscure and warrants further study

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Potentiation of Sham Feeding Response in Pavlov Pouch Dogs by Subthreshold Amounts of Gastrin with and without Acidification of Denervated Antrum

By

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Abstract

OLBE L *Potentiation of sham feeding response in Pavlov pouch dogs by subthreshold amounts of gastrin with and without acidification of denervated antrum* Acta physiol scand 1964 *61* 244—254 — The sham feeding response of Pavlov pouch dogs in which gastrin release had been suppressed by total denervation or resection of the antrum was augmented 4—21 times by i v injection of subthreshold amounts of gastrin demonstrating that subthreshold amounts of gastrin potentiated the effect of vagal stimulation of the HCl glands The sham feeding responses with or without potentiation by subthreshold amounts of exogenous gastrin were not inhibited by acidification of totally denervated antral pouches suggesting that the hypothetical antral chalone does not interfere at the parietal cell level with the cephalic phase of gastric acid secretion

Previous reports have shown that gastrin is released from the antrum by vagal stimuli (Uvnas 1942, Burstall and Schofield 1954 Oberhelman Jr Rigler and Dragstedt 1957 Woodward et al 1957, Maung Pe Thein and Schofield 1959 Nyhus et al 1960) and that gastrin and vagal stimulation of the HCl glands act synergistically during the cephalic phase of gastric acid secretion (Uvnas 1942 Olbe 1963) Furthermore Uvnas (1942) has suggested that even subthreshold amounts of gastrin may significantly augment the secretory response to vagal stimulation of the HCl glands The main purpose of the present study was to investigate the influence of subthreshold amounts of exogenous gastrin on the secretory response to sham feeding in dogs in which the release of gastrin was minimized

Antral acidification inhibits gastric acid secretion either by inhibition of gastrin release (Longhi et al 1957 Maung Pe Thein and Schofield 1959 Shapiro and State 1961) or by liberation of a chalone with antisecretory properties

(Shimizu Morrison and Harrison 1958 Danhof 1960 Thompson et al 1962) or by a combination of these two mechanisms Another purpose of the present study was to investigate whether antral acidification could interfere with the effect of subthreshold amounts of gastrin on the HCl glands

Methods

Operative Procedures

Ten healthy mongrel dogs (A B E—G I J M—O) weighing 14—20 kg were used The dogs were initially provided with an esophageal fistula (Olbe 1959) and a Pavlov pouch according to the method of Thomas (1942) and subsequently with an innervated antral pouch with a cutaneous fistula (Olbe 1963) During the second operation 3—4 cm of the mucosa proximal to the antrum corpus boundary and the duodenal bulb just proximal to the entrance of the common bile duct were resected in dogs M N and O in order to minimize gastrin release from regions other than the antral pouch At a third operation the antral pouch was totally denervated by separation of the antrum from the corpus as well as by transection of the major and minor omentum of the antrum leaving the pouch supplied by vessels from the abdominal wall At a fourth operation the totally denervated antral pouch was resected in dogs B J M—O

Gastrin Preparation

Gastrin was prepared from the antral mucosa of hogs according to the method of Gregory and Tracy (1961) However the purification was stopped after the first stage The secretory activity of the preparations was 42—240 histamine units (Uvnäs and Emås 1961) per mg gastrin The histamine content was less than 0 06 μg free histamine base per mg gastrin

Experimental Procedures

A recovery period of at least 2—3 weeks followed each operation Before each experiment the dogs fasted for 18—20 hours During the experiments the Pavlov pouch secretion was collected in 15 min periods starting with the fasting secretion for at least one hour and the amount of acid in the secretion was determined by titration against 0 01 N NaOH with phenolphthalein as indicator During sham feeding the esophageal fistula was opened and the dogs repeatedly reconsumed minced meat or fish for a period of 10 min. The secretory response to sham feeding was followed for $3^1/_2$ hours

The gastrin was dissolved in isotonic saline acidified to pH 3 The gastrin solution was infused i v in a concentration of 0 01—10 mg per ml at a constant rate of 4—13 ml per hour Each dog received the same gastrin preparation throughout the experimental series with one exception (see legend Fig 2) The sensitivity of the Pavlov pouches to gastrin was determined in dogs I M—O by infusing a subthreshold dose of gastrin intravenously for one hour and approximately doubling the dose every hour by changing gastrin concentration and/or rate of the infusion until a definite secretory response was obtained The secretory response to the infusion of each fixed dose of gastrin was expressed as the secretory output during the fourth 15 min period of the infusion During the sham feeding experiments infusion of subthreshold amounts of gastrin was started at least one hour before beginning the sham feeding and was subsequently continued for further $3^1/_2$ hours

Antral acidification was achieved with 0 1 N HCl according to a previously described method (Andersson and Olbe 1964) The acidification started 15 min before sham feeding and proceeded throughout the sham feeding experiment

Table I Effect of total denervation of the antrum and acidification to pH 1 of the totally denervated antral pouch on 3 5 hours sham feeding responses in Pavlov pouch dogs

	Mean sham feeding response					
	Before antral exclusion		After antral denervation		After antral denervation with antral acidification	
Dog	mEq ± S E	No of exp	mEq ± S E	No of exp	mEq ± S E	No of exp
A	2.04 ± 0.21	4	2.93 ± 0.14	3	3.00 ± 0.30	2
B	1.05 ± 0.20	4	1.87 ± 0.18	4	2.20 ± 0.54	3
E	3.26 ± 0.45	4	1.06 ± 0.23	15	1.07 ± 0.40	2
F	0.76 ± 0.13	4	0.83 ± 0.15	3	1.46 ± 0.10	3
G	1.30 ± 0.17	5	1.15 ± 0.34	8	1.03 ± 0.15	3
I	0.49 ± 0.07	5	0.24 ± 0.04	6	—	
M	1.37 ± 0.14	4	0.28 ± 0.07	5	0.49 ± 0.08	5
N	1.28 ± 0.27	4	0.10 ± 0.01	7	—	
O	0.68 ± 0.14	5	0.19 ± 0.05	6	—	

Local anesthesia of the totally denervated antral pouch was obtained by keeping a 2 per cent tetracaine solution in the pouch against an outflow pressure of about 10 cm of water The tetracaine was applied during one hour before sham feeding and during the first 15 min of the third hour after the start of sham feeding

Results

Before antral exclusion definite secretory responses to sham feeding were obtained in all 9 dogs (Table I) Following total denervation of the antral pouch the sham feeding responses were significantly reduced ($p < 0.01$ by the *t* test) in dogs E M N, and O (Table I) The sham feeding responses almost disappeared in dogs M N and O (Table I Fig 2) in which the duodenal bulb and distal part of the corpus had been resected in addition to the total denervation of the antral pouch

Effect of Subthreshold Amounts of Exogenous Gastrin on Sham Feeding Response After total denervation of the antral pouch (dogs I and M) and after resection of the antrum (dog B) the effect of i v infusion of varying subthreshold doses of gastrin on the sham feeding response was determined (34 expts) Two to four expts were performed with each dose of gastrin The subthreshold dosage of gastrin was determined by the dose response relationship for i v infused gastrin in dogs I and M (Table II) and was controlled in each sham feeding experiment (dogs B I M) by absent response to gastrin infusion for at least 1 hour before the sham feeding In dog B the available amounts of gastrin did not suffice to establish a dose response relationship A secretory response to i v infused gastrin was obtained by a dose of 0 32 histamine units per kg per min in dog I and by

Table II Pavlov pouch responses to intravenous infusion of varying doses of gastrin expressed as the secretory output during the 4th 15 min period of the infusion

Dog I

Dose of gastrin in histamine units/kg/min	Control Fasting secretion	0.039	0.058	0.179	0.315	0.579
Mean response mEq/15 min ± S E						
After denervation of antrum (3 exp)	0.01 ± 0	0.07 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.07 ± 0.02	0.16 ± 0.05

Dog M

Dose of gastrin in histamine units/kg/min	Control Fasting secretion	0.074	0.136	0.420	0.740	1.360
Mean response mEq/15 min ± S E						
After denervation of antrum (4 exp)	0	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.06 ± 0.02	0.20 ± 0.04
After resection of antrum (4 exp)	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.07 ± 0.09	0.20 ± 0.03	0.27 ± 0.05

Dog N

Dose of gastrin in histamine units/kg/min	Control Fasting secretion	0.160	0.419	0.890	1.597	2.814
Mean response mEq/15 min ± S E						
After denervation of antrum (4 exp)	0.01 ± 0	0.03 ± 0.01	0.03 ± 0.02	0.05 ± 0.01	0.12 ± 0.05	0.43 ± 0.13
After resection of antrum (4 exp)	0.01 ± 0	0.01 ± 0.01	0.05 ± 0.02	0.12 ± 0.04	0.19 ± 0.05	0.26 ± 0.05

Dog O

Dose of gastrin in histamine units/kg/min	Control Fasting secretion	0.334	0.614	1.897	3.342
Mean response mEq/15 min ± S E					
After denervation of antrum (3 exp)	0.01 ± 0	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.10 ± 0.03
After resection of antrum (1 exp)	0.01	0	0.01	0.07	0.24

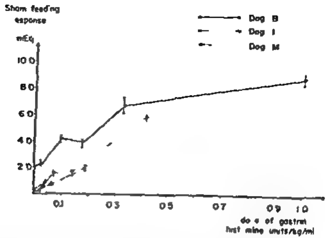

Fig 1 The effect of subthreshold doses of intravenously injected gastrin on mean 3 ½ hours sham feeding response in Pavlov pouch dogs with minimized release of gastrin ⟙ = range
The dose of gastrin that produced acid secretion was > 1 018 (dog B) 0 315 (dog I) and 0 740 (dog M) histamine units per kg per min (cf Table II)

0 74 histamine units per kg per min in dog M Dog B did not respond to 1 02 histamine units per kg per min A subthreshold dose of gastrin less than 1/5 of the dose producing acid secretion doubled the sham feeding response and with increasing subthreshold dose of gastrin the sham feeding response was further augmented up to 4—21 times (Fig 1) The sham feeding response most augmented by a subthreshold dose of exogenous gastrin in dogs B and I equalled the sham feeding response after exclusion of the vagally innervated antrum (Table III)

Effect of Acidification of Totally Denervated Antrum on Sham Feeding Response During sham feeding experiments 0 1 N HCl was instilled into the totally denervated antrum with (dogs J M) and without (dogs A B E—G) outflow pressure of 10 cm water No inhibitory effect was observed in dogs A B E—G or M (Table I) On the other hand the sham feeding response was markedly inhibited in dog J (Table IV) Further exceptional responses to sham feeding in dog J were observed after total denervation of the antral pouch increasing successively from the 13th postoperative week to the level found before antral denervation when the excluded antral pouch was vagally innervated (Table IV) Instillation of a local anesthetic into the antral pouch after total denervation had the same inhibitory effect as antral acidification and resection of antrum (Table IV) At operation the antral pouch which had been totally denervated was found to have almost completely reunited with the corpus

Effect of Acidification of Totally Denervated Antrum on Sham Feeding Response during Administration of Subthreshold Dose of Exogenous Gastrin The sham feeding response during i v infusion of a subthreshold dose of gastrin was determined in dogs I M—O with (16 expts) and without (16 expts) acidification to pH 1 of the

Table III Augmentation of mean 3 5 hours sham feeding response in Pavlov pouch dogs by vagal release of gastrin from innervated isolated antrum and by subthreshold amounts of exogenous gastrin in high dosage during minimized release of gastrin Dose of exogenous gastrin was 1 018 (dog B) and 0 179 (dog I) histamine units per kg per min (cf Fig 1 and Table II)

	Mean sham feeding response					
	After exclusion of vagally innervated antrum		After denervation (dog I) or resection (dog B) of antrum			
			without exogenous gastrin		with exogenous gastrin	
Dog	mEq ± S [redacted]	No of exp	mEq ± S E	No of exp	mEq ± S E.	No of exp
B	6 60 ± 0 31	[redacted]	2 07 ± 0.27	6	8 77 ± 0 46	2
I	1 84 ± 0.24	4	0.24 ± 0 04	6	1 96 ± 0 16	2

Table IV The exceptional 3 5 hours sham feeding responses in a Pavlov pouch dog (J) following total denervation of the antral pouch related to those before and after exclusion of the vagally innervated antrum and after resection of the antrum

Sham feeding response													
Mean response before antral exclusion		Mean response after exclusion of vagally innervated antrum		After antral denervation		After antral denervation with antral acidification to pH 1		After antral denervation with local anesthesia of the antrum		Mean response after resection of antrum	
mEq ±S E	No of exp	mEq ±S E	No of exp	mEq	Week after denervation	mEq	Week after denervation	mEq	Week after denervation	mEq ±S E	No of exp
0 50± 0 07	5	3 96± 0.23	4	0 54	12	0 43	11	0 19	20	0 68± 0 11	4
				0 47	12	0 71	12	0 20	20		
				1 07	13	0 96	14	1 00	21		
				0 91	13	0 31	14	0 30	23		
				2 24	13	0 14	15	0 77	25		
				3 15	15	Mean 0 51		Mean 0 49			
				2 60	16						
				4 63	16						
				3 40	16						

totally denervated antral pouch The subthreshold dose of gastrin was 0 236 (dog I) 0 136 (dog M) 0 160 (dog N) and 0 614 (dog O) histamine units per kg per min (cf Table II the gastrin preparation of dog I in this series of expts was however not the same as that used for determination of dose response

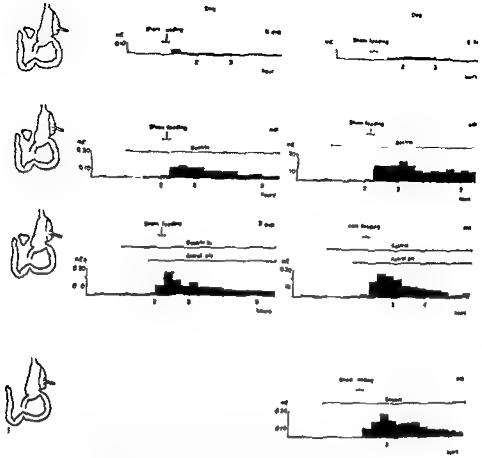

Fig 2A Effect of acidification or resection of totally denervated antral pouches on mean sham feeding responses augmented by subthreshold amounts of exogenous gastrin in Pavlov pouch dogs. Dose of gastrin in dog M was 0 136 histamine units per kg per min (cf Table II) Dose of gastrin in dog I was 0 236 histamine units per kg per min but the gastrin preparation was not the same as that used for determination of dose response relationship in Table II

relationship in Table II) Antral acidification was performed with an outflow pressure of 10 cm of water The subthreshold dose of gastrin increased the sham feeding response in all dogs (Fig 2) The gastrin augmented sham feeding response was unaffected by antral acidification in dog I and was slightly but not significantly reduced ($p > 0.01$ by the *t* test) in dogs M N and O The apparent reduction was most evident towards the end of the acidification period in dogs M and N (Fig 2) Comparing the sham feeding response during administration of the same dose of gastrin after resection of the antral pouch in dogs M—O (10 expts) showed that the gastrin augmented sham feeding response after resection of antrum was not significantly higher ($p > 0.01$ by the *t* test) than that during acidification of the totally denervated antrum The relatively

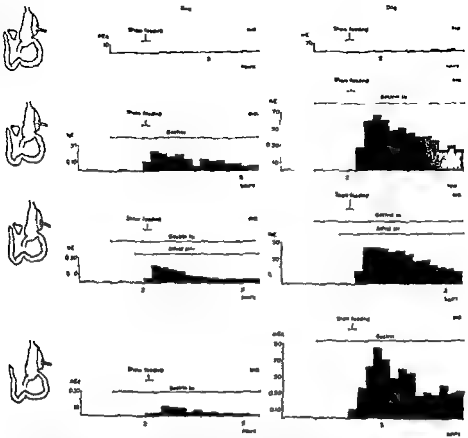

Fig. 2B Effect of acidification or resection of totally denervated antral pouches on mean sham feeding responses augmented by subthreshold amounts of exogenous gastrin in Pavlov pouch dogs. Dose of gastrin was 0 160 (dog N) and 0 614 (dog O) histamine units per kg per min (cf Table II)

low secretory rate previously noted towards the end of the acidification period in dogs M and N was also observed after resection of the antrum (Fig 2) The sensitivity to i v infused gastrin was the same both before and after resection of the antrum in dogs N and O and increased slightly after antrum resection in dog M (Table II)

Discussion

In the present study the sham feeding response in Pavlov pouch dogs was substantially augmented by i v injection of subthreshold amounts of exogenous gastrin after ensuring minimal release of the endogenous gastrin by antral denervation or resection The increase of the sham feeding responses depended on the

subthreshold dose of infused gastrin (Fig 1) showing that the effect of vagal stimulation of the HCl glands was markedly potentiated by subthreshold amounts of gastrin The results emphasize that very small amounts of endogenous gastrin may maintain a substantial nervous secretory response It is as yet uncertain whether the remaining sham feeding response in Pavlov pouch dogs after total denervation or resection of the antrum is dependent only on vagal impulses to the HCl glands or requires minute amounts of gastrin perhaps spontaneously released from the denervated antrum or relased from other regions The second alternative is supported by the abolishment of the sham feeding response after total denervation of the antral pouch as well as resection of the distal part of the corpus and the duodenal bulb (dogs M—O)

Maung Pe Thein and Schofield (1959) found that gastrin was released from the innervated neutralized antrum by vagal stimulation and Andersson and Olbe (1964) showed that the release of gastrin by vagal stimulation of an innervated antrum with antral pH $\geqq 2$ resulted in a substantial augmentation of the sham feeding response In the present study the potentiation of the sham feeding response by subthreshold amounts of exogenous gastrin in high dosage equalled that found during vagal release of gastrin from the neutral innervated antrum (Table III) The results suggest that an essential physiologic effect of vagally released gastrin might be to enhance the sensitivity of the HCl glands to vagal secretory impulses

The nervous secretory response is substantially inhibited by acidification of the vagally innervated antrum (for ref see Andersson and Olbe 1964) In the present study acidification to pH 1 of antral pouches which had been totally denervated did not inhibit the sham feeding responses in 6 of 7 Pavlov pouch dogs[1] The failure to elicit inhibition is probably not due to functional disintegration of the antrum after total denervation since the ability to release gastrin by mechanical stimulation is still retained (Olbe 1963) The results agree with the recent finding of Johnson (1963) that the secretory response to insulin hypoglycemia is not inhibited by acidification of vagally denervated antral pouches It is true Shimizu Harrison and Morrison (1958) and Thompson et al (1962) have reported that acidification of a denervated antrum inhibited the secretory response to insulin hypoglycemia but incomplete antral denervation and some degree of postoperative reinnervation by vagal fibres of the antrum could not be excluded in their experiments The results of the present study indicate the pre

[1] In the exceptional dog (dog J) postoperative restoration of vagal fibres to the antrum with subsequent vagal release of gastrin was suggested by the following reasons 1) The sham feeding respons successively increased during the fourth postoperative month to values greater than those before antral exclusion and similar to the post-exclusion values seen when the antrum was still innervated indicating increased vagal release of gastrin from the antrum (cf Olbe 1963 2) The sham feeding response was inhibited by local anesthesia of the antrum indicating suppression of gastrin release from the antrum (Woodward and Schapiro 1958 Nyhus et al 1960 3) At operation the antrum was found to be re united with the corpus thus providing the anatomical situation for regeneration or sprouting (Murray 1962 of vagal fibres to the antrum and 4) Resection of the antral pouch abolished the increase of the sham feeding response suggesting that vagal release of gastrin from the antrum caused the hypersecretion

sibility that release of even minute amounts of gastrin through remaining or restored vagal fibres to a "denervated" antrum may substantially augment the nervous secretory response and that acidification of such an antrum may elicit inhibition by suppression of vagal release of gastrin.

The liberation of a chalone with antisecretory properties by acidification of an innervated or denervated antrum has been suggested by the demonstration of inhibitory effect of blood from acidified antrum (Danhof 1960, DuVal Jr. and Price 1961, Thompson et al. 1962). The hypothetical antral chalone has been proposed to act at the parietal cell level (Thompson et al. 1962). It is unlikely that the chalone might counteract the secretory effect of vagal discharge on the HCl glands, since the secretory responses to insulin hypoglycemia (Johnson 1963) and sham feeding in the present study were unaffected by acidification of denervated antral pouches. Furthermore the chalone was unable to inhibit the effect of gastrin in a dosage which produced high secretory rates (Gillespie and Grossman 1962, Andersson and Olbe 1964). Still the antral chalone might have physiologic significance by inhibiting the effect of minute amounts of gastrin on the HCl glands. The present findings that acidification of a denervated antrum did not inhibit the effect of subthreshold amounts of gastrin combined with vagal discharge on the HCl glands constitute evidence against such a physiologic significance of a chalone liberated from the acidified antrum and acting at the parietal cell level. No significant inhibition occurred but a slight reduction of the acid output was noted towards the end of the acidification periods in 2 of 4 dogs (Fig. 2). This reduction was probably not caused by an antral chalone since it also occurred after resection of the antrum. The present results in addition to the recent finding (Andersson and Olbe 1964) that a small submaximal dose of exogenous gastrin eliminated the inhibitory effect by acidification of the vagally innervated antrum on the secretory response to vagal stimulation involving vagal release of gastrin from the antrum lend further support to the hypothesis of Longhi et al. (1957) that suppression of gastrin release from the antrum is the physiological mechanism by which antral acidification inhibits gastric acid secretion.

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Acta physiol scand 1964 61 255—271

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Gastric Acid Secretion in Gastric Fistula Cats before and after Vagotomy and in Vagotomized Gastric Fistula Cats during Reserpine Treatment[1]

By

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Abstract

EMÅS S *Gastric acid secretion in gastric fistula cats before and after vagotomy and in vagotomized gastric fistula cats during reserpine treatment* Acta physiol scand 1964 61 255—271 — In nonanesthetized cats with gastric fistulae reserpine injected daily for 3 days or more increased basal gastric secretion of acid and the acid secretory responses to intravenous histamine and gastrin The increases had in an earlier study been shown to persist after sympathectomy and the main purpose of the present study was to determine the effect of vagotomy on these increases Vagotomy significantly reduced the secretory responses of nonanesthetized cats to histamine and gastrin and after vagotomy reserpine treatment did not produce any increase of basal secretion or of the secretory responses to histamine and gastrin The latter finding proves that intact vagal innervation of the stomach is necessary for the development of hypersecretion during reserpine treatment Maximal subthreshold amounts of methacholine infused intravenously into vagotomized cats produced increases of the secretory responses to histamine and gastrin approximately equal to those produced by reserpine treatment in cats before vagotomy It is suggested that the reduction of the responses following vagotomy is due mainly to the elimination of the vagal tone which in the fasting cats is assumed to sensitize the parietal cells to histamine and gastrin The hypersecretory effects of reserpine treatment in cats with gastric fistulae and the absence of these effects after vagotomy demonstrates that reserpine treatment increases the sensitivity of the parietal cells to histamine and gastrin only in the vagally innervated stomach The increased sensitivity is supposed to be due to a centrally induced enhancement of vagal tone The increases in the secretory responses of vagotomized cats during the infusion of subthreshold amounts of methacholine are consistent with this explanation

Reserpine treatment of nonanesthetized cats with gastric fistulae for 3 days or more (0 10 to 0 15 mg per kg of b w i m) was shown to increase basal gastric acid secretion and the acid secretory responses to i v histamine and gastrin (Emås 1963) The hypersecretory responses of cats treated with reser

[1] Parts of this investigation were presented at the XXII International Congress of Physiological Sciences in Leiden Holland 1962 (Emås 1962)

pine suggested an increased excitability of the parietal cells which could be due either to a reduction or loss of inhibitory influences or to an increase of stimulating factors In a subsequent paper (Emås 1964) evidence was presented that the ability of reserpine to deplete catecholamines from adrenergic nerve endings (for references see Emås 1964) thereby reducing the sympathetic nerve influences on the stomach was not the cause of the hypersecretion in reserpine treated cats

One possible explanation mentioned for the hypersecretion of reserpine treated cats was that reserpine treatment might produce a parasympathetic predominance which increases the excitability of the parietal cells to histamine and gastrin (Emås 1963) The present study is directed toward this possibility the primary object having been to determine the influence of vagotomy on the hypersecretory effects of reserpine treatment During the course of the experiments the question arose whether vagal denervation of the stomach or the administration of a cholinergic drug in cats affect the excitability of the parietal cells to exogenous histamine and gastrin The investigation was therefore extended to include also the effect of vagotomy on the secretory responses to histamine and gastrin, and the effect of subthreshold amounts of methacholine on the secretory responses of vagotomized cats to histamine and gastrin

Methods

Operative Procedures

Nine cats (male and female) weighing 3 0 to 4 0 kg and provided with gastric cannulas (Emås 1960) were used In one animal subjected to vagotomy (no 71) the celiac ganglia and the distal parts of the thoracic sympathetic chains had been resected 22 and 14 months earlier (Emås 1964)

All operations were performed under sterile precautions with pentobarbital sodium anesthesia (Nembutal, Abbott 25 to 35 mg per kg of body weight i v) Artificial respiration was given during vagotomy (Emås 1964)

In 3 cats bilateral vagotomy was performed via a median abdominal incision The esophagus was freed from the diaphragm under slight downward traction the of stomach The peritoneum was incised ventrally from the cardia to the hiatus exposing the muscle layer of the distal esophagus The whole circumference of the esophagus was freed from adjacent tissues and 1 5 to 3 cm of the posterior and anterior vagal trunk were resected between nylon ligatures The diaphragm was attached to the esophagus by a few interrupted stiches In the remaining 6 cats bilateral vagotomy was performed through a thoracotomy between the 7th and 8th left rib With this approach the vagal trunks were easily localized under the pleura to the left and right of the esophagus Sometimes a thin branch of the right vagal trunk was found ventral to the esophagus The pleura was incised and 3 to 4 cm of the esophagus was dissected free Two to 4 cm of each nerve were resected between nylon ligatures The chest was then closed while the lungs were inflated Postoperative management was the same as after preganglionic symphathectomy (Emås 1964)

Vagotomy was considered complete when in two experiments insulin hypoglycemia elicited no acid secretory response (Emås 1963) despite a blood sugar level during max

imal hypoglycemia of less than 50 mg glucose per 100 ml blood The first experiment with insulin was carried out within 3 months after the operation and the second 2 weeks to 6 months later In one animal (no 86) 9 month and in another (no 71) 5 months elapsed between operation and the last experiment with insulin with no signs of vagal reinnervation By this criterion vagotomy was complete in all animals used in the study although 2 cats had to be reoperated before this was attained

In 2 out of [redacted] cats hair accumulated in the stomach as evidenced after 5 (cat 86) and [redacted] months (cat 48) by turbid and ill smelling gastric juice and elevation of basal gastric acid secretion The bolus of hair was removed through an 1 5 cm long gastrotomy in the corpus perpendicular to the minor curvature at the same time as a pyloroplasty (Heineke Mikulicz) was performed by an extramucosal myotomy This operation normalized the secretory pattern Postoperative management as described in a previous paper (Emås 1960)

Experimental Technique

Acid secretory response to insulin hypoglycemia was determined before vagotomy in all but one animal and after vagotomy in all animals according to the method described previously (Emås 1963) The dose of insulin was increased after vagotomy if this was necessary to secure a blood sugar level during maximal hypoglycemia of less than 50 mg glucose per 100 ml blood Regular insulin was given i v in doses ranging from 0 5 to 1 0 I U per kg of body weight

A Effect of Vagotomy on Gastric Acid Secretion 1 hour basal secretion and the secretory responses to histamine were determined before and after vagotomy in 7 cats (prevagotomy and postvagotomy controls) The corresponding responses to gastrin were determined in 6 cats.

B Effect of Reserpine (Serpedin©) Treatment on Gastric Acid Secretion in Vagotomized Cats 1 hour basal secretion and the secretory responses to either histamine or gastrin or both were determined in 8 vagotomized cats (postvagotomy controls) The experiments were repeated during periods of daily injections of reserpine

C Effect of Subthreshold Amounts of Methacholine (Betacholyl©) on the Acid Secretory Responses to Histamine and Gastrin in Vagotomized Cats The secretory responses to histamine and gastrin were determined in 3 vagotomized cats. The responses were re-determined during continuous i v infusion of methacholine in amounts which alone elicited no appreciable secretion of acid

In the control experiments and in the experiments performed during reserpine treatment histamine or gastrin was infused i v for 15 min every hour for 4 hours The principle for the experiments is apparent from Fig 1 and 2 for details see an earlier paper (Emås 1963) In the experiments with methacholine basal secretion was recorded for at least 4 15 minute periods Methacholine dissolved in physiological saline (0 9 per cent NaCl) was then continuously given i v for 4 hours by an infusion pump (infusion rate 0 11 ml per min) During the 3rd and 4th hour of methacholine administration histamine or gastrin was infused i v for 15 minutes (Fig 3 and 4)

Gastric juice was collected over 15-min periods and the output of free and total acid determined by titration against 0 01 N NaOH with Topfer s reagent and phenolphthalein as indicators

Basal secretion was defined as meq of total acid secreted during the hour preceding the infusion of histamine gastrin or methacholine and the responses to histamine and gastrin as meq of total acid secreted during 1 hour from the commencement of the infusion minus the 1 hour basal secretion (Emås 1963) or — in the experiments with methacholine — minus the mean 1 hour secretion during the 1st and nd hour of methacholine infusion

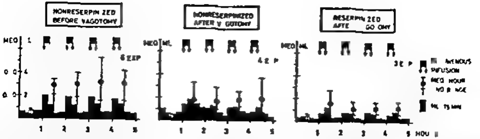

Fig 1 Basal gastric secretion and secretory responses (uncorrected) to repeated i v infusions of histamine in a gastric fistula cat (no 48) To the left nonreserpinized before vagotomy (mean of 6 expts) *in the middle* nonreserpinized after vagotomy (mean of 4 expts) and to the right reserpinized after vagotomy (mean of 3 expts) Dose of histamine 0 010 mg per kg of body weight for 15 min The secretory responses of the reserpinized animal before vagotomy are to be found elsewhere (Emås 1963 Table I)

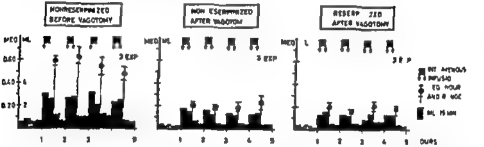

Fig 2 Basal gastric secretion and secretory responses (uncorrected) to repeated i v infusions of gastrin in a gastric fistula cat (no 41) The diagrams are arranged from left to right as described in the text to Fig 1 Each diagram represents the mean of 3 expts Dose of the gastrin 0 35 mg per kg of body weight for 15 min The secretory responses of the reserpinized animal before vagotomy have been illustrated elsewhere (Emås 1963 Fig ?)

The doses of histamine (as dihydrochloride) and of the gastrin preparations were adjusted to produce submaximal secretory responses as previously described (Emås 1963) Doses of histamine ranged from 0 010 to 0 030 mg per kg of body weight for 15 min and those of gastrin from 0 20 to 0 35 mg per kg The total dose was kept constant for each individual throughout the experimental series Animals used in previous investigations with reserpine (Emås 1963 1964) received the same total dose of histamine or gastrin in the present study The dose of methacholine (as iodide) used in each animal was the largest which just failed to evoke acid secretion or produced only traces of acid (maximal subthreshold dose) The doses administered ranged from 0 5 to 1 5 μg per min

Gastrin was prepared from antral mucosa of hogs by a somewhat shortened modification (Emås 1960) of the method devised by Jorpes *et al* (1952) Two gastrin preparations were used (3p and 9p) one (3p) having a secretory activity of 50 histamine units (Lynäs and Emås 1961) per mg preparation and the other (9p) 100 histamine units per mg The preparations are identical with those used in previous studies with reserpine (Emås 1963 1964) and contained no measurable amounts of histamine (< 0 1 μg of histamine dihydrochloride per mg preparation)

Table I Mean 1 hour basal secretion and mean 1 hour secretory response to intra encus infusions of histamine in gastric fistula cats before and after bilateral vagotomy

		Before vagotomy			After vagotomy			Decrease	
		Secretion in meq total acid			Secretion in meq total acid				
Cat no	Histamine in mg/kg	No of exp	Basal secretion	Response[1] to histamine (1st infusion)	No of exp	Basal secretion	Response[1] to histamine (1st infusion)	In meq total acid	In per cent of mean for prevagotomy controls
29	0.010	5	0.02	0.27 0.19– 0.37	5	0.01	0.12 0.09– 0.15	0.15	56
41	0.010	5	0.04	0.46 0.41– 0.55	4	0.0[illegible]	0.13 0.05– 0.20	0.33	72
48	0.010	6	0.05	0.23 0.15– 0.28	4	0.04	0.15 0.04– 0.31	0.08	35
94	0.015	5	0.0[illegible]	0.32 0.[illegible]8 0.37	5	0.01	0.25 0.18– 0.29	0.07	[illegible]
98	0.020	8	0.01	0.39 0.33– 0.48	5	0.01	0.10 0.08– 0.14	0.29	74
110	0.030	7	0.0[illegible]	0.44 0.29– 0.53	4	0.01	0.11 0.09– 0.13	0.33	75
Pre and postganglionically sympathectomized									
71	0.015	8	0.0[illegible]	0.43 0.33– 0.57	5	0.01	0.41 0.31– 0.52	0.02	5

Correction made for basal secretion.
Range of secretory responses.
Illustrated in Fig 1
Illustrated in Fig 3

Reserpine Treatment

Reserpine in a dose of 0.10 to 0.15 mg per kg of b w was injected i m once daily for periods of usually 9 to 15 days (mean 12 days) Secretory studies started after 3 days treatment with reserpine after which period the animal is referred to as reserpinized

Table II *Mean 1 hour basal secretion and mean 1 hour secretory response to intravenous infusions of gastrin in gastric fistula cats before and after bilateral vagotomy*

			Before vagotomy			After vagotomy				
			Secretion in meq total acid			Secretion in meq total acid			Decrease	
Cat no	Gastrin prep no	Gastrin in mg/kg	No of exp	Basal secretion	Response to gastrin (1st infusion)	No of exp	Basal secretion	Response[*] to gastrin (1st infusion)	In meq total acid	In per cent of mean for prevagotomy controls
*41	3p	0 35	3	0 02	**0.57** 0 54–0 60	3	0 01	**0 21** 0 18–0 23	**0 36**	63
48	3p	0 35	3	0 02	**0 26** 0 23–0 29	3	0 02	**0 18** 0 08–0 24	**0 08**	31
*94	9p	0 20	5	0 01	**0 40** 0 32–0 45	5	0 00	**0 25** 0 19 0 28	**0 15**	[illegible]
98	9p	0 25	4	0 01	**0.34** 0 23–0 43	4	0 01	**0 03** 0 02–0 04	**0.31**	91
110	9p	0.20	5	0 01	**0 42** 0 36–0 52	5	0 01	**0 10** 0 08 0 13	**0 32**	75
Pre and postganglionically sympathectomized										
71	3p	0 25	4	0 03	**0.51** 0 44–0 59	4	0 01	**0 41** 0 35 0 50	**0 10**	20

Correction made for basal secretion
* Illustrated in Fig 2
Range of secretory responses
* Illustrated in Fig 4

(Emås 1963) and were then repeated every 3rd or 4th day The last reserpine injection was given 20 to 24 hours before each experiment One or two periods of reserpinization were carried out on each animal For further details see Emås (1963

Evaluation of Data

The statistical methods used in previous studies with reserpine (Emås 1963 1964) were applied *To estimate the effects of vagotomy and of reserpine treatment on basal*

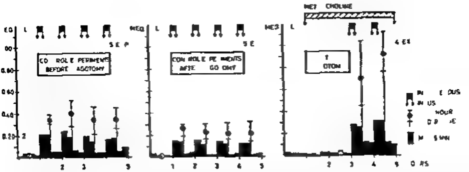

Fig 3 Basal gastric secretion and secretory responses (uncorrected) to repeated i v infusions of histamine in a gastric fistula cat (no 94) To the left before vagotomy (mean of 5 expts) in the middle after vagotomy (mean of 5 expts.) and to the right after vagotomy but during continuous i v infusion of maximal subthreshold amounts of methacholine (mean of 4 expts) Dose of histamine 0 015 mg per kg of body weight for 15 min Dose of methacholine 1 0 or 1 5 µg per min

acid secretion the rank sum test (Dixon and Massey 1957) was used while their effects on secretory responses to histamine and gastrin were evaluated by a modified procedure for analysis of variance (Snedecor 1956 Chapt 12 Sections 14 and 15)

Results

A Effect of Vagotomy on Gastric Acid Secretion

The control experiments did not start until at least 2 weeks after vagotomy and were completed within a period of 3 to 7 months

Basal Secretion (Tables I and II)

Titratable amounts of free acid were rare in the prevagotomy controls (68 expts) and the mean 1 hour basal output of total acid for individual cats varied between less than 0 01 and 0 04 meq

After vagotomy the mean output of total acid (56 expts) in all 7 cats was less than 0 03 meq The rank sum test (Dixon and Massey 1957) revealed no significant difference ($P > 0.05$) between total acid output before and after vagotomy in 5 animals In one cat (no 41) vagotomy reduced basal secretion almost significantly ($P < 0.05$) and in the remaining cat (no 71) the reduction was significant ($P < 0.01$)

Secretory Responses to Histamine (Table I)

The mean secretory responses obtained in the control experiments before vagotomy on cats no 41 48 and 71 are given elsewhere (Emas 1963 1964) Those from cats no 48 and 94 are illustrated in Fig 1 and 3 respectively In the remaining 3 cats control experiments revealed in principle the same secre

Table III Mean 1 hour basal secretion and mean 1 hour secretory responses to 4 repeated intravenous infusions of histamine in vagotomized gastric fistula cats nonreserpinized and reserpinized

				Nonreserpinized (controls)				Reserpinized						Decrease (1st infusion)	
			Secretion in meq total acid						Secretion in meq total acid						
				Response¹ to histamine infusion no						Response¹ to histamine infusion no					
Cat no	Histamine in mg/kg	No of exp	Basal secretion	1	2	3	4	No of exp	Basal secretion	1	2	3	4	In meq total acid	In per cent of mean f...
29	0 010	5	0 01	0 12 ²0 09– 0 15	0 11	0 08	0 10	2	0 05	0 12 ²0 03– 0 21	0 04	0 05	0 04	0 00	0
30	0 010	4	0 01	0 22 0 14– 0 28	0 20	0 27	0 21	4	0 02	0 15 0 08– 0 21	0 16	0 19	0 16	0 07	32
41	0 010	4	0 02	0 13 0 05– 0 20	0 13	0 19	0 13	2	0 01	0 08 0 04– 0 12	0 08	0 09	0 07	0 05	39
³ ⁴48	0 010	4	0 04	0 15 0 04– 0 31	0 13	0 15	0 16	3	0 03	0 13 0 04– 0 21	0 09	0 11	0 10	0 02	13
8G	0 030	6	0 03	0 22 0 13 0 45	0 19	0 20	0 15	3	0 02	0 20 0 11– 0 36	0 19	0 23	0 21	0 02	9
91	0 015	5	0 01	0.25 0 18– 0 29	0.21	0.20	0 20	4	0 02	0 21 0 08– 0 32	0 21	0 20	0.23	0 04	16
Pre and postganglionically sympathectomized															
⁵71	0 015	5	0 01	0 41 0 31– 0 52	0 33	0.29	0 30	3	0 01	0.39 0 32 0 51	0 42	0 39	0 40	0 02	5

Correction made for basal secretion

² Range of secretory responses

Corresponding experiments have been performed before vagotomy (Emås 1963 Table I)

⁴ Illustrated in Fig 1

⁵ Corresponding experiments have been performed before vagotomy (Emås 1964 Table I) and before vagotomy and sympathectomy (Emås 1963 Table I)

tory pattern as in the cats mentioned above and in those used in previous studies (Emås 1960 1963) Table I therefore gives only the mean response to the first histamine infusions in each animal

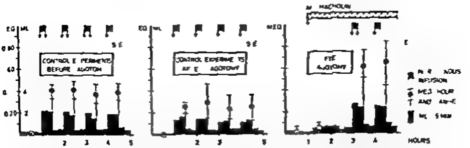

Fig 4 Basal gastric secretion and secretory responses (uncorrected) to repeated i v infusions of gastrin in a gastric fistula cat (no 94) The diagrams are arranged from left to right as described in the text to Fig 3 The number of experiments and the doses of methacholine are the same as in Fig 3 Dose of the gastrin preparation 0.20 mg per kg of body weight for 15 min

After vagotomy hourly repeated histamine infusions produced secretory responses (Fig 1 and 3 Table III) which were as uniform as before (Emås 1963 1964) Vagotomy reduced the mean response to the first infusions in all 7 animals (Table I) the mean reduction ranging from 0 02 meq of total acid or 5 per cent (cat 71) to 0 33 meq (cats no 41 and 110) or 75 per cent (cat 110) Responses before and after vagotomy differed significantly ($P < 0\ 01$) according to the analysis of variance (Snedecor 1956) The experimental data obtained from cat 71 were excluded from this analysis as both sympathectomy and vagotomy had been performed on this animal The effect of vagotomy on the mean responses of cat 48 is illustrated in Fig 1 and on those of cat 94 in Fig 3

Secretory Responses to Gastrin (Table II)

The mean responses in the prevagotomy controls on cat 41 and cat 94 are shown in Fig 2 and 4 respectively For corresponding data on cats no 48 110 and 71 the reader is referred to previous papers (Emås 1963 1964) The secretory pattern of cat 98 was similar to that of the other cats and — as with histamine — only the mean response of each individual to the first gastrin infusions is therefore given in Table II

After vagotomy the responses to repeated gastrin infusions (Fig 2 and 4 Table IV) were as constant as those to histamine The mean response to the first infusions decreased in all 6 cats (Table II) For individual cats the mean reduction varied from 0 08 meq of total acid (cat 48) to 0 36 meq (cat 41) and the percentage reduction from 20 (cat 71) to 91 (cat 98) According to the analysis of variance (data on cat 71 omitted) there was a significant difference ($P < 0\ 01$) between the responses before and after vagotomy Fig 2 and 4 demonstrate the effect of vagotomy on the mean secretory responses of cats no 41 and 94 respectively

Table II Mean 1 hour basal secretion and mean 1 hour secretory responses to 4 repeated intravenous infusions of gastrin in vagotomized gastric fistula cats nonreserpinized and reserpinized

			Nonreserpinized (controls)						Reserpinized						Decrease (1st infusion)	
				Secretion in meq total acid						Secretion in meq total acid						
					Response[1] to gastrin infusion no						Response to gastrin infusion no					
Cat no	Gastrin prep no	Gastrin in µg/kg	No of exp	Basal secretion	1	2	3	4	No of exp	Basal secretion	1	2	3	4	In meq total acid	In per cent of mean of controls
*41	3p	0.35	3	0.01	**0.21** *0.18– 0.23	0.17	0.18	0.23	3	0.02	**0.18** 0.12– 0.23	0.14	0.19	0.16	**0.03**	14
48	3p	0.35	3	0.02	**0.18** 0.08– 0.24	0.24	0.23	0.18	2	0.01	**0.24** 0.20– 0.28	0.15	0.18	–	**–0.06**	33
85	9p	0.35	4	0.02	**0.28** 0.25– 0.35	0.30	0.25	0.37	3	0.04	**0.21** 0.01– 0.33	0.23	0.20	0.17	**0.07**	25
94	9p	0.20	5	0.00	**0.25** 0.19– 0.28	0.29	0.22	0.24	4	0.01	**0.11** 0.07 0.15	0.14	0.13	0.15	**0.14**	[illegible]
110	9p	0.20	5	0.01	**0.10** 0.08– 0.13	0.13	0.14	0.11	4	0.02	**0.10** 0.06– 0.13	0.11	0.13	0.12	**0.00**	0
Pre and postganglionically sympathectomized																
71	3p	0.25	4	0.01	**0.41** 0.35– 0.50	0.34	0.31	0.27	4	0.00	**0.25** 0.14 0.32	0.20	0.18	0.18	**0.16**	39

[1] Correction made for basal secretion
[2] Corresponding experiments have been performed before vagotomy (Emås 1963 Table II)
Illustrated in Fig 2
Range of secretory responses
[5] Mean of 2 responses
Corresponding experiments have been performed before vagotomy Emås 1964 Table II)

B Effect of Reserpine Treatment on Gastric Acid Secretion in Vagotomized Cats

In 2 cats reserpine treatment was instituted one month after complete vagotomy had been attained in 5 cats 3 to 4 months and in one cat (no 86) 10 months after vagotomy Most control experiments were carried out before reserpine treatment started

Basal Secretion (Table III and IV)

In the nonreserpinized state 57 expts were carried out on 8 cats The mean 1 hour basal output of toal acid was in each animal less than 0 04 meq

During reserpinization (41 expts) free acid in the gastric juice was observed only rarely in the vagotomized cats and the mean output of total acid by individual animals did not exceed 0 05 meq per hour According to the rank sum test the basal output of total acid in the postvagotomy controls and in the experiments during reserpinization did not differ significantly (P > 0 05) in any animal

Secretory Responses to Histamine (Table III)

The experiments were performed on 7 cats 3 of which (no 41 48 and 71) had been used in similar experiments with reserpine before vagotomy (Emås 1963 1964)

As in the previous studies (Emås 1963 1964) the effect of reserpinization on the secretory responses was calculated from the response to the first infusion only in each control and in each experiment during reserpine treatment Reserpinization had no influence on the mean secretory response of cat 29 In the remaining 6 vagotomized cats reserpinization slightly reduced the mean response the reduction being in the order of 0 02 to 0 07 meq of total acid Mean responses in the experiments on cat 48 are shown in Fig 1 Analysis of variance revealed no significant difference (*P* > 0 05) between the secretory responses of the animals nonreserpinized and reserpinized The data from cat 71 were not included in the analysis for the reason mentioned above

Secretory Responses to Gastrin (Table IV)

Of the 6 animals included in this study 5 were also used in the experiments with histamine The effect of reserpinization on secretory responses to gastrin had been investigated before vagotomy in cats no 41 48 110 (Emås 1963) and 71 (Emås 1964) though the latter animal was symphatectomized

The effect of reserpinization on mean secretory responses varied in different cats The mean response to first infusions increased during reserpinization by 0 06 meq in cat 48 remained unchanged in cat 110 and decreased in the remaining 4 cats by 0 03 to 0 16 meq The mean responses of cat 41 are illustrated in Fig 2 Analysis of variance (data of cat 71 omitted) showed that the responses of the cats nonreserpinized and reserpinized did not differ significantly (*P* > 0 05) after vagotomy

Five cats succumbed in connection with reserpinization cat 1 one day cat 86 two days and the remaining cats (no 29 41 and 110) three days after the last reserpine injection Cats no 41 and 86 were lost for postmortem examination In cat 71 two small ulcers were situated in the distal part of the antrum and a larger one 5 mm in diameter at the approximate antrum-corpus border In cat 29 three elongated mucosal erosions 20 mm in length were found in the distal part of the body of the stomach but no mac

Table IV Mean 1 hour basal secretion and mean 1 hour secretory responses to 4 repeated intravenous infusions of gastrin in vagotomized gastric fistula cats nonreserpinized and reserpinized

Cat no	Gastrin prep no	Gastrin in mg/kg	Nonreserpinized (controls)						Reserpinized						Decrease (1st infusion)	
				Secretion in meq total acid						Secretion in meq total acid						
			No of exp	Basal secretion	Response[1] to gastrin infusion no 1	2	3	4	No of exp	Basal secretion	Response[1] to gastrin infusion no 1	2	3	4	In meq total acid	In per cent of mean for controls
[2,3]41	3p	0.35	3	0.01	0.21 [4]0.18–0.23	0.17	0.18	0.23	3	0.02	0.18 [4]0.12–0.23	0.14	0.19	0.16	0.03	14
[6]48	3p	0.35	3	0.02	0.18 0.08–0.24	0.24	0.23	[5]0.18	2	0.01	0.24 0.20–0.28	0.15	0.18	–	0.06	–33
85	9p	0.35	4	0.02	0.28 0.25–0.35	0.30	0.25	0.37	3	0.04	0.21 0.01–0.39	0.23	0.20	0.17	0.07	25
94	9p	0.20	5	0.00	0.25 0.19–0.28	0.29	0.22	0.24	4	0.01	0.11 0.07 0.15	0.14	0.13	0.15	0.14	56
[6]110	9p	0.20	5	0.01	0.10 0.08–0.13	0.13	0.14	0.11	4	0.02	0.10 0.06–0.13	0.11	0.13	0.12	0.00	0
[7] Pre and postganglionically sympathectomized																
71	3p	0.25	4	0.01	0.41 0.35 0.50	0.34	0.31	0.27	4	0.00	0.25 0.14 0.32	0.20	0.18	0.18	0.16	39

[1] Correction made for basal secretion
[2] Corresponding experiments have been performed before vagotomy (Emås 1963 Table II)
[3] Illustrated in Fig 2
[4] Range of secretory responses
[5] Mean of 2 responses
[6] Corresponding experiments have been performed before vagotomy (Emås 1964 Table III)

B Effect of Reserpine Treatment on Gastric Acid Secretion in Vagotomized Cats

In 2 cats reserpine treatment was instituted one month after complete vagotomy had been attained in 5 cats 3 to 4 months and in one cat (no 86) 10 months after vagotomy Most control experiments were carried out before reserpine treatment started

ceeded that in the postvagotomy controls (Table II) by 0 38 meq or 93 per cent and that in the prevagotomy controls (Table II) by 0 28 meq or 55 per cent

For individual cats the mean output of total acid during 2 hours of methacholine infusion alone amounted to 0 05 meq per hour or less

Discussion

Vagotomy reduced the acid secretory responses to histamine and gastrin in all cats The magnitude of the reduction varied considerably from animal to animal but in the same individual a large reduction in the responses to histamine corresponded to a large reduction to gastrin Similar studies on non-anesthetized cats have not been reported but in dogs vagal denervation of total stomach pouches (Oberhelman and Dragstedt 1948 Lewis 1951) and vagal denervation of the whole stomach in multiple gastric pouch preparations (Ferguson 1953) and gastric fistula preparations (Antia *et al* 1951) has been shown to reduce the acid secretory responses to histamine. The effect of vagal denervation of the whole stomach on responses to exogenous gastrin has not previously been investigated In most of the studies on dogs referred to above (Antia *et al* 1951 Lewis 1951 Ferguson 1953) vagotomy reduced basal acid secretion In the present investigation basal secretion of acid was slightly reduced by vagotomy in 6 of 7 cats but the reduction was significant only in 1 animal probably due to the small basal output of acid before vagotomy

Vagal impulses and gastrin act synergistically on the parietal cells in cats (Uvnas 1942) and dogs (Olbe 1963) and synergism has also been demonstrated in dogs between local cholinergic reflexes elicited by distension of the stomach and injected gastrin and histamine (Grossman 1961 a) Similarly subthreshold amounts of cholinergic drugs potentiate the secretory responses to endogenous gastrin in dogs (Gregory and Tracy 1960) and to exogenous gastrin and histamine in dogs (Grossman 1961 b Langlois and Grossman 1950) and cats (present investigation) These findings suggest that the susceptibility of the parietal cells to histamine and gastrin is increased by concomitant cholinergic excitation and conversely the reduced secretory responses to histamine and gastrin after vagotomy indicate a decreased susceptibility of the parietal cells to these stimuli This decreased susceptibility may be attributable to the elimination of the vagal tone assumed to sensitize the parietal cells to histamine and gastrin in the fasting cat Passaro Gillespie and Grossman (1963) have very recently reported that exogenous gastrin potentiates the secretory action of histamine in dogs Since vagal impulses release gastrin (for references see Schofield 1960) both vagal impulses and released gastrin may in the fasting cat sensitize the parietal cells to histamine The reduction of the secretory responses to histamine after vagotomy may accordingly be due not only to the elimination of subthreshold vagal impulses to the HCl-secreting cells but also to the reduction or block of gastrin release.

It has been established in previous investigations that prolonged reserpine treatment produces basal hypersecretion of acid gastric juice and greatly increases the secretory responses to histamine and gastrin both in ordinary gastric fistula cats (Emås 1963) and in sympathectomized gastric fistula cats (Emås 1964). The present experiments demonstrate that reserpinization fails to produce these effects in vagotomized cats. Four of the 8 cats studied here had been subjected to reserpine treatment before vagotomy (Emås 1963, 1964) and since reserpinization then produced hypersecretion, it was possible to conclude that bilateral vagotomy eliminated the hypersecretory effects of reserpinization.

The fact that vagal innervation of the stomach is indispensable for the hypersecretion of reserpinized cats strongly suggests a central action of reserpinization. It has been argued that reserpine produces a parasympathetic predominance, either by depressing the central sympathetic activity (Bein 1953, 1957) or by activating the central parasympathetic system (Brodie, Prockop and Shore 1958). Since cholinergic excitation increases the sensitivity of the parietal cells to histamine and gastrin, the increased secretory responses to histamine and gastrin in reserpinized cats with intact vagal innervation could be ascribed to increased vagal tone sensitizing the parietal cells to these stimuli. This interpretation explains why, in the vagotomized cats, reserpinization has no effect on the secretory responses to histamine and gastrin. The findings that histamine and gastrin produce approximately as large secretory responses of acid in vagotomized cats continuously infused with maximal subthreshold amounts of methacholine as in ordinary gastric fistula cats treated with reserpine may indicate that methacholine and reserpinization sensitize the parietal cells to about the same degree. Another mechanism, however, may also be implicated in potentiating the secretory action of histamine. Vagal impulses (Schofield 1960) and cholinergic drugs (Langlois and Grossman 1950, Burstall and Schofield 1954) are considered to release gastrin and, according to Passaro *et al.* (1963), gastrin potentiates the secretory action of histamine. Supposing vagal impulses in the reserpinized ordinary gastric fistula cats and the amounts of methacholine infused in the vagotomized cats to be capable of releasing endogenous gastrin, the gastrin released and the cholinergic excitation of the parietal cells could both potentiate the action of histamine. A release of endogenous gastrin does not, however, explain the potentiation by reserpinization or methacholine of the secretory action of exogenous gastrin.

Previous experiments (Emås 1963) had shown that reserpinization of ordinary gastric fistula cats did not alter the secretory responses to methacholine and reduced those to insulin hypoglycemia. The action of these stimuli involves at least two components: release of endogenous gastrin and increase of the excitability of the parietal cells to the released gastrin. In reserpinized cats with intact vagal innervation the parietal cells exhibit an increased excitability to gastrin, presumably due to an enhanced vagal tone. The failure of reserpiniza-

tion to increase the secretory responses to methacholine and insulin hypoglycemia may suggest (1) that the increased excitability of the parietal cells to gastrin in the reserpinized cats is not further increased by methacholine or vagal impulses or/and (2) that these stimuli release lesser amounts of gastrin in reserpinized than in nonreserpinized cats (Emås 1963)

Several explanations are possible for the elevated basal acid secretion in ordinary gastric fistula cats treated with reserpine (1) It was suggested above that reserpinization increases vagal tone In the reserpinized cats the vagal impulses may therefore be capable of activating the HCl secreting cells (2) As vagal impulses release gastrin (Schofield 1960) the increased vagal tone may result in a release of endogenous gastrin or in an acceleration of spontaneous gastrin release the action of the released gastrin then being potentiated by vagal impulses (3) Injected reserpine causes a transient reduction of the gastrin activity in the antral mucosa of cats (Emas and Fyro 1963) suggesting gastrin release The action of the liberated gastrin is then potentiated by vagal impulses It is not known however whether the release of gastrin is still continuing 20 to 24 hours after the injection of reserpine the time at which secretory studies started or whether a single reserpine injection does release gastrin in reserpinized cats (4) Reserpine reduces the histamine content of different organs including the gastrointestinal tract (Waalkes Coburn and Terry 1959 Haverback and Wirtschafter 1962 Kim and Shore 1963 Moran and Westerholm 1963) although no evidence has been published of histamine release in cats under prevailing experimental conditions An action of liberated histamine similar to that of gastrin has however, to be considered as a possibility None of these explanations of the basal hypersecretion of reserpinized cats can be excluded on the basis of our present knowledge although this study has demonstrated the outstanding role played by the vagal nerves The finding that vagotomy and atropine (Emås 1962) block the basal hypersecretion of reserpinized cats does not invalidate any of the explanations proposed

Subthreshold amounts of methacholine potentiated the secretory action of exogenous histamine and gastrin in the vagotomized cats The responses exceeded those evoked by histamine and gastrin alone before vagotomy and approximated the responses of ordinary gastric fistula cats treated with reserpine These findings suggest that methacholine (in maximal subthreshold amounts) in vagotomized cats and reserpinization in ordinary gastric fistula cats made the parietal cells more sensitive to histamine and gastrin than did vagal tone in ordinary gastric fistula cats

Although the number of observations is small it is interesting to note that while vagotomy eliminated the hypersecretory effects of reserpine treatment it did not prevent the occurrence of gastric ulcers

To sum up the present investigation has demonstrated (1) that vagotomy reduces the secretory responses of gastric fistula cats to exogenous histamine and gastrin (2) that vagotomy eliminates the hypersecretory effects of reser

pinization, and (3) that maximal subthreshold amounts of methacholine in vagotomized cats potentiate the secretory action of exogenous histamine and gastrin. The lowered responses after vagotomy reflect a reduced excitability of the parietal cells. The reduction is supposed to be due mainly to the elimination of vagal tone assumed in the fasting cats to sensitize the HCl secreting cells. The hypersecretion of ordinary gastric fistula cats treated with reserpine reflects an increased excitability of the parietal cells. The increase is supposed to be due to a centrally induced enhancement of vagal tone sensitizing the HCl secreting cells to histamine and gastrin. The secretory responses to histamine and gastrin were about as large in vagotomized cats continuously infused with methacholine as in reserpinized cats with intact vagal innervation. This suggests that methacholine in vagotomized cats and reserpinization in ordinary gastric fistula cats caused greater sensitization of the parietal cells to exogenous histamine and gastrin then was caused by vagal tone in ordinary gastric fistula cats.

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Acta physiol scand 1964 61 272—278

From the Department of Physiology Karolinska Institutet Stockholm Sweden

Effect of Vagotomy, Vagus Stimulation and Various Drugs on the Substance P Content in the Small Intestine of the Rabbit

By

B Radmanovic[1]

Received 4 December 1963

Abstract

Radmanovic B *Effect of vagotomy vagus stimulation and various drugs on the Substance P content in the small intestine of the rabbit* Acta physiol scand 1964 61 272—278 — Cervical vagotomy significantly increased the Substance P (SP) content in the rabbit s small intestine from 2 8 ± 0 2 U/g to 7 2 ± 0 6 U/g Stimulation of either right or left vagus in the neck significantly decreased SP in the same tissue from 2 8 ± 0 2 to 1 0 ± 0 2 U/g The SP values increased 15 min after cessation of stimulation and reached control values within 30 min Physostigmine significantly decreased the SP content in the rabbit s intestine to 1 7 ± 0 2 U/g while no definite effect was observed after administration of hexamethonium chlorisondamine atropine reserpine chlorpromazine p hydroxymercuribenzoate or nicotine It is suggested that vagus stimulation and physostigmine d pl te the SP stores in some peripheral nervous structures in the intestine Conversely vagus section may allow a replenishing of the stores

Since the basic investigation on the properties of substance P (SP) by Euler and Gaddum (1931) numerous experiments have been made in order to obtain more knowledge about its chemical properties and possible physiological role in the organism Besides the actions on smooth muscle SP exerts some central effects (for references see Symposium on Substance P Sarajevo) It has also been suggested that it acts as a transmitter substance between the first and second sensory neurones (Lembeck 1959) and as a motility hormone in the digestive tract (Euler 1936) The distribution of SP in this area has been studied in detail by Douglas et al (1951) and by Pernow (1953)

It has recently been reported that SP occurs in the sediment from homogenized guinea pig brain (Lembeck and Holasek 1960) Similar results have been obtained by Cleugh and Whittaker (quoted by Gaddum 1961) Inouye and Kataoka (1962) have described subcellular distribution of SP in the central

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nervous system SP also occurs in subcellular particles in peripheral nerves in amounts from 1 5—6 U/g (Euler and Lishajko 1961 Euler 1963)

The intestinal smooth muscle is stimulated both by a direct action (Pernow 1953) and by a peripheral neurotropic effect i e stimulation of afferent fibres in the peristaltic reflex arc (Beleslin and Varagic 1958) However the functional relationship if existing is not clear between the SP present in the nervous tissue and its action on the smooth muscle of the intestinal wall By studying the effect of vagus nerve stimulation and denervation upon the amount of SP in the intestinal wall it was hoped to obtain more knowledge on this point The amounts of SP in the intestine after administration of some drugs which affect the intestinal motility were also measured

Material and methods

In the present experiments rabbits of both sexes were used weighing from 2 to 3 kg

Vagotomy was performed both in the neck and subdiaphragmatically under ether anesthesia Bilateral vagotomy was performed in two steps with a 4 days interval between the operations Twentyfour hours after bilateral vagotomy the rabbits were sacrified and SP extracted from the intestine

The divided right or left vagus in the neck were stimulated by bipolar silver electrodes covered with cotton wool soaked in warm liquid paraffine A stimulator giving square wave pulses (Grass model S 4) was us d The frequency was 5 imp/sec voltage 10 V and pulse duration 0 5 msec The total duration of stimulation was 20 min

The drugs used were atropine sulfate hexamethonium bromide chlorisondamine chloride (Ecolid Ciba) reserpine (Serpasil Ciba) chlorpromazine chloride (Hibernal Leo) p-hydroxymercuribenzoate physostigmine salicylate and nicotine hydrogen tartrate

Preparation of extracts The extracts were made from the small intestine. In some experiments SP was separately extracted from duodenum and ileum. In the other experiments SP was obtained from the whole of the small intestine

Intestine was removed immediately after the animal was killed by a blow on the neck flushed with Tyrode solution and cut into small pieces ($^1/_2 \times {}^1/_2$ cm) with scissors and boiled for 10 min in 5 volumes of distilled water acidified with sulfuric acid to pH 4 SP remains stable at pH 4 even during prolonged boiling (Gaddum and Schild 1934) After cooling and filtering the extract was saturated with ammonium sulfate (Euler 1936) and left for 12 hours at 2 C After filtration the dried precipitate was dissolved in about 10 ml water and methanol added in small portions to 70 % v/v with stirring After settling at 2 C for 12 hours the voluminous precipitate was centrifuged off and discarded The clear supernatant (about 30 ml) was passed through a 3 cm high column of aluminium oxide (diameter of column 2 cm flow 1 ml per min) The column was eluted with 15 ml portions of methanol in water in decreasing concentrations of 70 % 50 % and 25 followed by repeated elutions with water according to Pernow (1953)

After removing the methanol by evaporation in vacuo the eluates were used for bioassay on the isolated guinea pig ileum A 3 ml bath was employed containing atropine sulfate and promethazine in concentrations of 0 4 mg per l and LSD in a concentration of 0 1 mg per l Several extracts were also tested on the chicken rectal caecum in a 10 ml bath

As standard a preparation manufactured by Hoffmann La Roche containing 75 U/mg was used The recovery was checked and the mean recoveries were of the order of 75

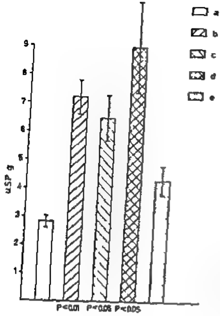

Fig 1 Each block represents amount of SP from the rabbit s small intestine in units per gram of tissue a control b SP amount after unilateral vagotomy on the right side in the neck c after unilateral vagotomy on the left side d after sh rt lasting (24 hours) bilateral vagotomy and e SP amount aft r subdiaphragmatic vagotomy Time after vagotomy 4 days

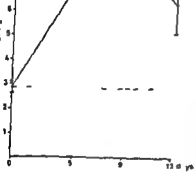

Fig 2 SP amount (U SP g) in the rabbit s small intestine after unilateral vagotomy in the n ck on the right side SP was extracted 5 th 9 th a d 13 th day after vagotomy (full l ne) Broken line control SP values

Results

Since no significant difference was found in the amount of the SP i ous part of small intestine of the rabbit (3 2±0 4 U/g in d um 0 2 U/g in ileum) the content was calculated per g of r
The value was 2 8 U g when tested on the isolated gu

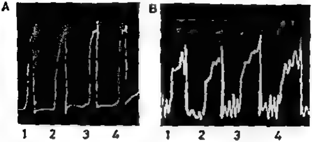

Fig 3 A Guinea pig ileum 3 ml bath (1) 1 U SP standard (2) 0.2 ml water I eluate of SP extract (3) 0.4 ml water II eluate (4) 0.5 ml water III eluate
B Chicken rectal caecum 15 ml bath (1) 0.5 U SP standard (2) 0.1 ml water I eluate of SP extract (3) 0.25 ml water II eluate (4) 0.2 ml of water II eluate

to atropine promethazine and LSD When rabbit's small intestine was cut into larger pieces (3 × 3 cm) only about 1 U/g of SP was obtained

SP in the rabbit's intestine after vagotomy As shown in Fig 1 the amounts of SP in the rabbit's gut were significantly increased after vagotomy SP increased to about the same extent after unilateral vagotomy (either on the right or on the left side) as after two-stage bilateral vagotomy in the neck The SP content in the extract from the rabbit's gut after subdiaphragmatic vagotomy was lower and not significantly increased probably due to incomplete surgical section of the vagi

The amounts of SP in the rabbit's gut reached a peak 9 days after unilateral vagotomy On the 13th day SP content was slightly decreased (Fig 2)

Some extracts from the rabbit's small intestine were tested on the chicken rectal caecum These extract showed a stimulating effects on the chicken rectal caecum corresponding to the amount of SP found on the guinea pig ileum when tested against the standard (Fig 3)

SP content in the rabbit's intestine after vagal stimulation In a series of experiments the peripheral parts of the cut vagus nerves were stimulated in the neck for 20 min Immediately after this period the animals were sacrificed and extracts prepared from the intestine As shown in Fig 4 the amount of SP in the rabbit's small intestine decreased from 2.8 to 1.0 U/g ($p < 0.01$) during the 20 min stimulation of the vagi It can also be seen that the effect of stimulation of the vagi is only transitory Fig 4 demonstrates that SP values had increased 15 min after cessation of stimulation and reached control values within 30 min

SP content in the rabbit's intestine after administration of various drugs Fig 5 shows the amounts of SP in the rabbit's whole small intestine after administration of the drugs in comparison with the control value All drugs in the present experiments were injected s.c. with the exception of nicotine which was administered i.p. Atropine sulfate was given for 3 days in a dose of 1—1.5 mg/kg/day Rabbits receiving hexamethonium bromide were given 4 mg/kg twice daily for 3 days Of chlorisondamine chloride (Ecolid Ciba) 2—2.5 mg/kg was injected

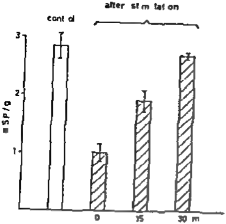

Fig 4 SP amounts (U per gram of tissue) in the rabbit s small intestine after stimulation of both vagus nerves in the neck

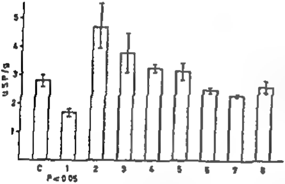

Fig 5 SP amounts (units per gram of tissue) in the small intestine of rabbit after administration of some drugs C control values Next columns represent SP amount after administration of (1) physostigmine salicylate (2) hexamethonium bromide (3) chlorisondamine chloride (4) atropine sulfate (5) reserpine (6) chlorpromazine (7) p-hydroxymercuribenzoate and (8) nicotine hydrogen tartrate Vertical bars S E M

... daily for 3 days Reserpine (0 5 mg/kg) chlorpromazine chloride (5 mg/kg) p-hydroxymercuribenzoate (10 mg/kg) and physostigmine salicylate (4 mg/kg) were given once a day for 3 days The dose of nicotine hydrogen tartrate employed was 4 mg and rabbits were sacrificed at the time of maximum miosis

Of all drugs examined in these experiments only physostigmine had a significant effect on the SP content in the rabbit s intestine After 3 days administration of physostigmine s c the SP content decreased from $2\,8 \pm 0\,2$ U/g in control experiments to $1\,7 \pm 0\,2$ U/g ($p < 0\,05$) After ganglionic blocking drugs the SP content in rabbit s gut showed a tendency to increase Atropine nicotine and drugs (reserpine chlorpromazine and p-hydroxymercuribenzoate) which release catecholamines from tissues did not change the amount of SP in the rabbit s small intestine

Discussion

The amounts of SP found in the rabbit s intestine with the purification technique used are considerably smaller than those found in previous studies (*Euler* 1936), the higher degree of purification in the present experiments may partly

account for the difference. Since the recovery of SP standard was about 75 % the actually found values should be increased by 25 %. In the present experiments the values are uncorrected since internal standard was not regularly used.

In previous studies the highest amounts of SP were found in the submucosa of the intestinal wall (Pernow 1953). Also in studies on cases of Hirschsprung's disease (Ehrenpreis and Pernow 1953) it was found that the amount of SP was small in the distal aganglionic segment of the sigmoid. These investigations suggest a close correlation between the nerve supply of the intestinal wall, its content of SP, and its peristaltic activity. As previously mentioned, SP has been demonstrated in subcellular particles of the vagus nerve (Euler 1963).

In pharmacological studies it has been observed that SP also acts on nervous structures in the intestinal wall. The effect of SP on the circular muscle on the isolated guinea pig ileum can be blocked by hexamethonium (Beleslin and Varagic 1958) as well as by morphine and morphine-like analgesics (Medakovic and Radmanovic 1959). It has been found also that SP can restore peristaltic activity of the isolated guinea pig ileum previously abolished by d-tubocurarine (Radmanovic 1961).

The present experiments show a marked increase on the SP content in the intestinal wall of the rabbit after unilateral and shortlasting bilateral vagus section. On the other hand stimulation of the vagus nerves causes a decrease of SP in the rabbit's intestine. The present results may be explained by assuming that vagus stimulation depletes the stores of SP in nervous structures in the intestinal wall. Conversely vagus section may allow a replenishing of the stores in a way similar to that observed for noradrenaline in skeletal muscles after section of preganglionic nerves (Sedvall 1963).

The experiments reported here also show that synthesis of SP is rather fast since the SP content in the intestine increased by about 65 per cent during 30 min after cessation of vagal stimulation.

I wish to express my sincere gratitude to professor U. S. v. Euler under whose guidance this work was carried out. This study was supported by a grant from Gustaf and Tyra Svenssons Foundation.

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Acta physiol scand 1964 61 279—298

From the Department of Clinical Physiology Karolinska sjukhuset Stockholm Sweden

Mechanical Systole at Rest, During and After Exercise in Supine and Sitting Position in Young and Old Men

By

T Strandell

Received 4 December 1963

Abstract

Strandell, T *Mechanical systole at rest during and after exercise in supine and sitting position in young and old men.* Acta physiol scand 1964 *61* 279—298 — In 12 healthy men aged 20—25 years and 20 aged 61—83 years mechanical systole was measured by phonocardiography as the time interval between the 1st and the 2nd sound During exercise with stepwise increased work loads mechanical systole at first decreased approximately linearly with increasing heart rate but at high heart rates a significant curvilinearity was recorded with successively lesser decrease of mechanical systole At low heart rates mechanical systole was shorter in sitting than in supine position but at high heart rates it was of the same duration in both positions. At rapidly increasing or decreasing heart rates the changes in duration of systole were slower than the changes in diastole The regression line of mechanical systole on heart rate was at a slightly but significantly higher level in the old than in the young men whereas the slope was similar The lower maximal heart rates during exercise in the old men were thus not accompanied by comparable changes in the mechanical events of the heart cycle However in the old men the maximal heart rate was probably significantly correlated to the slope of mechanical systole on heart rate The slope was also negatively correlated to the cardiac index at rest in the old men and the level of the line was negatively correlated to the systolic pressure increase in the brachial artery during exercise

The variations in the duration of mechanical ventricular systole has long been studied both in animals and man In general the systole shortens fairly regularly with decreasing length of heart cycle During sympathetic stimulation or injection of sympathomimetic amines however systole shortens in excess of the decrease in cycle length (Wiggers and Katz 1920 Kjellberg Rudhe and Sjöstrand 1951 Braunwald Sarnoff and Stainsby 1958) During progressive increase in venous return the systole at first increases in duration as the filling pressure and stroke volume increase (Wiggers and Katz 1920 Katz 1921 Wiggers 1921 and others) When the filling pressure is still further raised the stroke volume becomes smaller (decompensatory stage) and the systole shortens During hypothermia the systole is prolonged (Hegnauer Shriber

Table I. Some anthropometric data on 12 young and 20 old men. W_{130} = intensity of work at heart rate = 130. W_{max} and HR_{max} = work load and heart rate at maximal working intensity. n = number of individuals. Mean ± SD are given

n	Age (years)	Weight (kg)	Height (cm)	W_{130} (kpm/min)	W_{max} (kpm/min)	HR_{max} (beats/min) Sitting	Supine
12	22.8±1.2	67.5±8.0	180.9±6.3	656± 93	1,248±129	187.9±11.3	176.2±15.0
20	70.7±6.6	73.6±9.9	174.0±5.9	657±158	832±171	156.9±11.7	138.0±17.3

and Haterius 1950), as also during markedly increased aortic pressure (Wiggers 1921, Braunwald, Sarnoff and Stainsby 1958).

In man the systolic ejection phase at rest was found to be approximately linearly related to the total cycle length (Lombard and Cope 1926). For a given heart rate the systole was shorter in standing and sitting than in recumbent position. In that study no effect of age was observed. Michel (1960) however, observed a significant increase with age of the systolic ejection phase at rest in the supine position in a material of 409 men aged 20—70 years without changes in heart rate with rising age.

The effect of exercise on mechanical systole was studied by Holmgren (1956) who observed a linear decrease with increasing heart rate during supine exercise. The duration of systole was calculated from right ventricular pressure tracings at heart rates between 50—150 beats/min. Bevegård (1963) found by phonocardiography a linear increase of the inverted value of mechanical diastole on heart rate during exercise. This would indicate that mechanical systole was fairly linearly related to heart rate at low heart rates, but that the relationship was markedly curvilinear at higher heart rates with successively smaller decrease of systole for a given increase in heart rate.

The purpose of the present investigation was to make a further study of the relationship between mechanical systole and heart rate, especially the effect of circulatory changes due to differences in body position and age and the relationships to the central circulation. It was also thought to be of interest to study whether or not variations in the relationship between mechanical systole and heart rate could be associated with the decrease of the maximal heart rate during exercise with rising age or with the interindividual variations in maximal heart rate within a certain age group.

Material

The material consisted of two groups, one of young and one of old men. The young men included 12 healthy medical students aged 20—25 years. The group of old men comprised 20 healthy men aged 61—83 years who were randomly selected from a somewhat larger group. The selection and examination of the old men has been reported earlier (Strandell 1963, 1964 a). Some anthropometric data are given in Table I.

Methods and procedure

A measure of the duration of left ventricular mechanical systole was obtained from the time interval on the phonocardiogram between the first vibrations of the 1st and 2nd heart sounds at the nominal frequencies of the phonocardiograph filters of 100 c/sec or 400 c/sec. This time interval thus includes the isometric contraction phase and the systolic ejection phase. Mechanical diastole was calculated as the time interval between the 2nd and the 1st sound, and heart rate from the sum of systole and diastole. Mean values were calculated from at least 5, usually consecutive, heart cycles. The phonocardiograms were recorded over the 4th left intercostal space with a direct writing ink jet recorder (Mingograf 42 + 42 B, Elema, Järnh, Stockholm) at a paper speed of 100 mm/sec. During exercise the amplification was decreased to 1/2 or 1/5 of the value at rest.

The group of young men was studied twice. On one occasion the phonocardiogram was recorded at rest supine, at rest sitting on a bicycle ergometer (Holmgren and Mattsson 1954), and during sitting exercise at successively increased work loads until exhaustion. The loads were always 300—600—900 ... kpm/min. On the other occasion the recordings were performed only in supine position — at rest, during exercise, immediately after, and 1, 2, 3, 4, 5, 6 and 10 min after exercise. The relative order of the two tests was randomly distributed. During exercise recordings were made both after 1 and [illegible] min at each load.

The group of old men was studied only in supine position, at rest and during and after exercise. The loads were always 300—600 ... kpm/min except for cases 32, 43 and 47, when they were 200—400—600 ... kpm/min. In 6 of the subjects the recordings during exercise were only made after 5 min at each load, and in 3 subjects no recordings were made after exercise. The recordings after exercise were not as regularly timed as in the young men, and generally the last recordings were made 3—4 min after exercise.

The phonocardiograms were recorded either during free breathing (most of the subjects in the group of old men) or immediately after the respiration was stopped in normal expiration (all subjects in the group of young men). The effect of this held respiration on mechanical systole and diastole was studied in 6 of the young men at rest and during exercise in supine and sitting positions by comparing the 1st recorded heart beat with the 5th. There was no difference in duration of mechanical systole of probable significance either at rest or during exercise in sitting or supine position. For values at rest and during exercise counted together the difference in supine position was $+ 0.05 \pm 1.25$ csec (mean $\pm$ SD, $n = 32$, $P > 0.95$) and in sitting position $+ 0.06 \pm 1.12$ csec ($n = 27$, $P > 0.95$). The mechanical diastole, however, was 4.7 csec $\pm$ 5.4 (mean $\pm$ SD, $n = 12$, 2 observations per individual) longer in the 5th than in the 1st beat at rest in supine position ($P < 0.05$), whereas the difference during supine exercise was not even of probable significance ($+ 0.4 \pm 2.2$ csec, $n = 20$, $P > 0.4$). In sitting position there was no difference of even probable significance at rest but during exercise at 300 kpm/min the difference was $+ 5.6 \pm 4.2$ csec ($n =$ [illegible], $P < 0.05$). For all values in sitting position counted together the difference was $+ 0.7 \pm 1.0$ csec ($n = 27$, $P > 0.4$). The abovementioned differences should be of no significance for the observations in the present study. They would indicate that in the group of young men the recorded heart rates during held respiration were around 1.4 beats/min lower at rest supine than what might be expected during free respiration without changes in the duration of systole. During supine exercise they would indicate an insignificant decrease in heart rate of around 0.5 beats/min. For all the values in sitting position they would correspond to an insignificant decrease in heart rate of around 0.5 beats/min without changes in the duration of systole.

Table II Heart rate (beats/min) and mechanical systole (Mech syst csec) at rest (1st value in 12 young and 20 old men Heart rate at maximal working intensity is given as the last value

Case no (Age years)	Supine Heart rate	Supine Mech syst	Sitting Heart rate	Sitting Mech syst
I	67	32.7	65	29.2
(21)	105	28.5	[illegible]	27.1
	118	24.8	112	23.7
	140	18.9	124	19.7
	172	17.9	170	16.2
			193	15.6
	177		193	
II	63	34.7	64	31.6
(21)	101	26.8	104	26.6
	120	23.5	130	21.8
	151	20.2	155	18.2
	176	16.7	171	17.2
	178		185	
III	57	36.9	66	31.2
(22)	97	29.4	89	27.1
	114	25.6	123	22.8
	161	18.2		
	161		180	
IV	60	35.0	67	30.3
(22)	92	29.6	96	26.4
	111	26.0	115	22.5
	135	22.6	139	21.3
			165	18.6
	160		169	
V	59	33.2	60	29.9
(22)	102	29.1	97	27.7
	114	25.5	125	21.5
	149	19.1	152	17.8
			184	16.4
	152		187	
VI	69	33.9	[illegible]	31.6
(23)	113	27.1	95	28.7
	151	20.7	136	21.7
	180	18.3	180	18.4
			203	16.4
	197		203	
VII	66	31.3	66	29.3
(23)	104	27.7	91	28.0
	118	25.4	116	24.4
	149	20.6	137	21.9
			169	17.1
	173		185	
VIII	85	30.4	93	[illegible]
(23)	112	27.3	112	25.7
	129	25.3	121	22.9
	160	22.1	152	21.2
			190	17.6
	183		203	
IX	63	34.5	61	33.1
(23)	107	30.0	91	29.5
	117	26.0	114	25.3
	135	22.5	127	22.8
	168	20.4	157	21.3
	168		181	
X	62	36.0	64	31.7
(21)	92	31.0	87	29.6
	121	23.5	122	23.5
	163	17.7	158	19.2
			189	16.4
	192		189	
XI	85	28.6	75	27.5
(21)	115	[illegible]	106	23.6
	137	22.9	131	20.9
	160	20.6	165	17.3
	199	16.6	190	16.0
	200		204	
XII	65	33.5	64	29.0
(25)	93	[illegible]	88	29.3
	120	25.1	120	23.1
	156	20.9	147	[illegible]
	173		[illegible]	

for each individual) and during exercise (after 5 min at each load) in supine and sitt position for each individual

Case no (Age years)	Supine Heart rate	Mech. syst	Case no (Age years)	Supine Heart rate	Mech syst	Case no (Age years)	Supine Heart rate	Mech syst
8 (61)	60 89 111 131	37 3 33.2 28.3	43 (67)	57 96 109 123 145 156	33 5 29.8 27.5 25 7 22 3	66 (73)	69 100 140 144	33 5 28 0 27 0
14 (62)	64 129 162 163	34.8 25 7 22 1	45 (69)	[illegible] 105 127 150	37 5 30 1 25 0	70 (75)	68 97 116 138	34.8 30 5 26.9
22 (63)	80 127 145 153	32 5 24.5 21.5	47 (68)	90 111 125 132	30 0 27 5 24 5	74 (80)	81 100 129 131	33 1 30 0 24.8
[illegible] (65)	61 90 109 128 128	36 0 28.9 25.2 22 9	50 (70)	47 74 99 117	38 0 31 4 26.5	77 (81)	59 92 113 113	34.3 31.5 28 0
28 (66)	74 111 138 173 173	31 7 26 7 23 7 20 5	52 (72)	49 79 102 116	35.9 33 4 28.2	8 (81)	58 84 117 121	36.8 30 8 24 0
3 (66)	58 100 128 1 8	34.8 29 4 4.5	54 (71)	67 95 127 150 150	34 2 29 9 24.5 1.8	79 (83)	66 103 118	33.8 27 4
33 (68)	71 107 1 8 148	3 5 27.8 23 3	57 (72)	56 93 1 0 158 158	35.0 30 1 5 1 21 5			

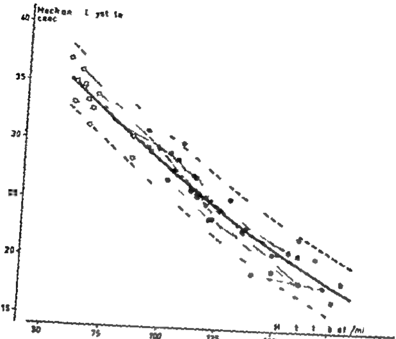

Fig 1 Mechanical systole in relation to heart rate at rest and during exercise in supine position in 12 young men Regression line (eq 2 Table III) ± 95 confidence belts

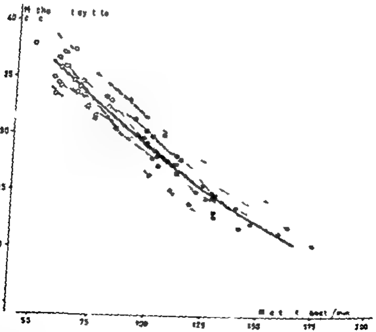

Fig 2 Mechanical systole in relation to heart rate at rest and during exercise in supine position in 20 old men (eq 10 Table III) Regression line ± 95 confidence belts

Table III Mechanical systole (y) in csec in relation to heart rate (x) in beats/min in 12 young and 10 old men n = number of observations Sup = supine position Sit = sitting position R = rest E = during exercise after 5 min at each load b = regression coefficient

Equation no	Age group (years)	n	Body position	Condition	Range for heart rate	Regression equation	Residual SD (csec)	b/s_b
1	20–25	59	Sup	R+E	57–199	$y=43.0-0.146x$	1.37	28.2***
2	20–25	52	Sup	R+E	57–199	$y=47.3-0.227x+0.000340x^2$	1.27	7.73 * / 2.79**
3	20–25	40	Sup	R+E	57–149	$y=44.7-0.165x$	1.21	23.2*
4	20–25	12	Sup	R	57–85	$y=48.2-0.221x$	1.28	5.24***
5	20–25	58	Sit	R+E	60–203	$y=38.2-0.119x$	1.35	27.1 *
6	20–25	58	Sit	R+E	60–203	$y=42.3-0.193x+0.000291x^2$	1.27	7.24*** / 2.78**
7	20–25	41	Sit	R+E	60–147	$y=39.7-0.136x$	1.28	17.4*
8	20–25	12	Sit	R	60–93	$y=37.2-0.103x$	0.56	5.40 *
9	60–83	65	Sup	R+E	47–173	$y=44.2-0.151x$	1.26	29.0
10	60–83	65	Sup	R+E	47–173	$y=48.5-0.243x+0.000441x^2$	1.16	7.95 * / 3.80***
11	60–83	69	Sup	R+E	47–150	$y=44.9-0.160x$	1.17	28.9 **
12	60–83	70	Sup	R	47–90	$y=45.2-0.163x$	1.26	5.70

The work test and the methods for determination of heart volume blood volume intensity of work at heart rate 130 (W130) work load and heart rate at maximal working intensity arterial lactate concentration during exercise degree of heart rate steady state during exercise and the assessment of the electrocardiographic changes during and after exercise have been described elsewhere (Strandell 1963 1964 a b c and d)

Most statistical calculations were performed according to Snedecor (1959) Differences between regression lines were tested according to Hald (1961) The following probability (P) levels were used $P < 0.001$*** = highly significant $P < 0.01$** = significant and $P < 0.05$* = probably significant

Results

Effect of heart rate

The individual values for mechanical systole at rest and during exercise (after 5 min at each load) are given in Table II The regression of mechanical systole at rest and during exercise on heart rate is shown in Fig 1 and 2 and Table III Assuming a linear decrease of mechanical systole with rising heart rate gave equations 1 5 and 9 (Table III) In the different groups however there was a significant curvilinearity (Fig 1 2 3) The relationship was then expressed as a second degree polynomial with a significant regression coefficient for (heart rate)² (eq 2 6 and 10 in Table III) giving a slight reduction of the

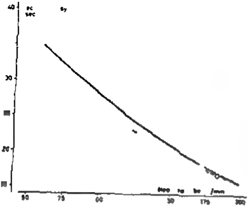

Fig 3 Mechanical systole in relation to heart rate at rest and during exercise in supine (whole line) (eq 2 Table III) compared with sitting body position (broken line) (eq 6 Table III) in 12 young men Mean values at maximal or near maximal working intensity in supine (●) and sitting position (○) are also given

residual standard deviation If the values obtained at heart rates above 150 beats per min were excluded from the calculations there was no significant regression coefficient for (heart rate)^2 ($P > 0.3$) and the straight regression lines were therefore calculated (eq 3, 7 11 in Table III)

A positive correlation was observed between the slope of mechanical systole on heart rate (x) and heart rate at maximal working intensity (y) (see below) A study was therefore made to see whether or not the curvilinearity of mechanical systole on heart rate (Fig 1 2) was due to different level and slope of straight individual curves or to curvilinearity of the individual curves For each individual a straight regression line was drawn through all values of mechanical systole on heart rate except the one with the highest heart rate In sitting position 11 of the 12 last individual observations of the young men during exercise fell above the straight regression lines and only 1 below According to the calculated values of chi square (6 75) this indicates a significant individual curvilinearity For the young men in supine position 9 of the last observations during exercise fell above the regression lines and 3 below which does not indicate curvilinearity ($P > 0.1$) For the observations in sitting and supine position together the chi square was significant 9 4 ($P > 0.005$) In the old men in supine position 9 of the last observations fell above the regression line and 10 below e g no indication of curvilinearity However in the old men the number of observations per individual was fewer and the conclusions therefore less valid

In all the groups studied the regression of mechanical systole on heart rate at rest (eq 4 8 12 in Table III) was not different from that of mechanical systole on heart rate using values both at rest and during exercise up to heart rate 150 ($P > 0.05$) When a regression line was computed for values only during exercise up to heart rate 150 the resting values in the group of old men did not differ from the extension of this line In the young men however the resting values had significantly shorter mechanical systoles both in supine (-1.6 ± 1.3 csec) (mean $\pm$ SD) and sitting position (-1.4 ± 1.5 csec)

Table IV Heart rate (HR beats/min) and mechanical systole (MS msec) at maximal or near maximal working intensity in 12 young and 20 old men Mean ± SD are given

Young men Supine		Young men Sitting		Old men Supine	
HR	MS[1]	HR	MS	HR	MS
173.1 ± 14.9	18.5 ± 1.5	183.9 ± 14.8	16.8 ± 1.2	131.5 ± 19.4	24.3 ± 2.6

[1] In 5 cases recorded after less than 5 min at the load
In 6 cases recorded after less than 5 min at the load
In 4 cases recorded after less than 5 min at the load

Effect of body position

At rest in sitting position the slope of the regression of mechanical systole on heart rate in the group of young men was flatter (less negative) than in supine position ($P < 0.02$) (eq 4 8 in Table III) When values during exercise up to heart rate 150 were included (eq 3 7 in Table III) the slope was significantly flatter in sitting than in supine position ($P < 0.01$) At higher heart rates the curved regression line for all the values in sitting position approached the curved line for the values in supine position (Fig 3) The extensions of the lines intersected at around heart rate 200 However the lines were rather uncertain at higher heart rates as only four observations were made above heart rate 170 in supine and only four above 190 beats per min in sitting position

At maximal or close to maximal working intensity in the young men the mechanical systole was significantly shorter and the heart rate significantly higher in sitting than in supine position (Table IV) The mean values were close to the regression lines (Fig 3) The difference in mechanical systole at maximal working intensity was thus entirely due to the difference in heart rate

Effect of age

At all heart rates but especially above 130 beats/min the regression line of mechanical systole on heart rate was at a slightly higher level in the old than in the young men (Fig 4) This difference in level was significant for the straight lines calculated for values at or below heart rate 150 ($P < 0.01$) whereas the slopes did not differ ($P > 0.8$) At higher heart rates a comparison between the lines is less meaningful as only 4 out of 20 of the old subjects were represented above 145 beats per min

At maximal or close to maximal working intensity the mechanical systole was shorter and the heart rate higher in the young than in the old men (Table IV) The mean values were close to the regression lines (Fig 4) The difference

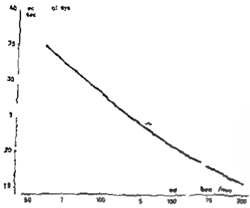

Fig 4 Mechanical systole in relation to heart rate at rest and during exercise in supine position in 1? young (whol line) (eq 2 Table III) compared with 20 old men (broken line) (eq 10 Table III) Mean values at maximal or near maximal working intensity in the young (●) and the old men (○) are also given

in mechanical systole at maximal working intensity with age was thus mostly connected with the lower heart rates in the old men Only a minor fraction of the difference was due to difference in level with age

Both for the young and the old men in supine position the mean mechanical diastole was shorter than the systole at maximal or near maximal working intensity (2 3 and 3 0 csec respectively)

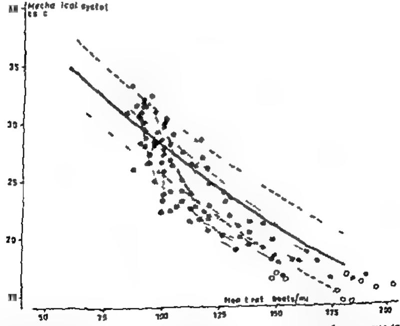

Fig 5 Mechanical systole in relation to heart rate in 12 young men after exercise in supine (●) and sitting position (○) Regression line for values at rest and after 5-min exercise at the load ± 95% confidence belts are given as in Fig 1 Dotted line denotes regression line for values after 1 min exercise at the load y = 44 7 − 0 158x SD = ± 1 14 n = 40 symbols as in Table III

Effect of duration of the work load

The regression lines for mechanical systole after 1 min exercise in supine position at the different loads in relation to heart rate are given for young and old men in Fig 5 and 6 The slopes of these lines were the same as for the lines calculated for values after 5 min (heart rate ≤ 150) The 1 min lines were however on a higher level ($P < 0.05$) with a mean difference of mechanical systole of 0 8 csec in the group of young men and 0 7 csec in the group of old men For the young men in sitting position the higher level for the 1 min line compared with the 5 min line was not significant ($p < 0.1$) the mean increase in systole being 0 7 csec

The individual values after 1 min exercise were compared with the straight lines through the preceding and succeeding 5 min values plotted against heart rate In the young men 29 out of 41 observations in supine position were above this line and so were 34 out of 49 observations in sitting position For the old men corresponding figures were 22 out of 29 According to the calculated values of chi square this indicates that in all the groups the 1 min values showed longer mechanical systoles in relation to heart rate than the 5 min values ($P < 0.05$) For the observations in all the groups together the difference is highly significant ($P < 0.001$) This indicates that under the circum

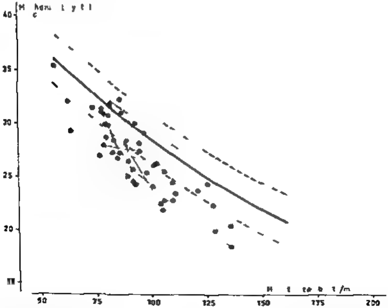

Fig 6 Mechanical systole in relation to heart rate after exercise in supine position in 17 old men (●) Regression line for values at rest and after 5-min exercise at the load ± 95 confidence belts are given as in Fig 2 Dotted line denotes regression line for values after 1 min exercise at the load $y = 44 - 0.14\,x$, $SD = 1.47$ $n = 9$ symbols as in Table III

Table V Total and some partial correlation coefficients (r) between the slope of mechanical systole on heart rate in supine position (individual regression coefficient 1), heart rate at maximal working intensity (2) 2—6 min heart rate increase at heart rate 130 (3) and assessment of ectopic beats during exercise test (4) in 20 men aged 61—83 years

Total r	Partial r	Partial r
$r_{12} = 0.41$	$r_{12.3} = 0.49^{*}$	$r_{23.14} = -0.57^{*}$
$r_{13} = -0.01$	$r_{12.4} = 0.50^{*}$	$r_{24.13} = -0.31$
$r_{14} = 0.21$	$r_{12.34} = 0.54^{*}$	
$r_{23} = -0.57^{**}$	$r_{13.24} = 0.26$	
$r_{24} = -0.29$	$r_{14.23} = 0.35$	

stances studied, the shortening of systole was slower than the heart rate increase during exercise The condition was not significantly influenced by body position age or heart rate within the studied heart rate range

Comparison between mechanical systole after and during exercise

The individual values for mechanical systole in relation to heart rate after exercise in supine position are given in Fig 5 and 6 The increase of the duration of mechanical systole with decreasing heart rate followed a curvilinear, not logarithmic course

Compared with the findings during exercise the heart rate at first decreased quicker than the systole increased Later the mechanical systole increased more steeply and 4 min after exercise all values in the group of young men were within the 95 % confidence belts for values at rest and during exercises The findings were essentially the same in the old men although they were not studied as closely as the young (see Methods and Procedure) The prolongation of mechanical systole continued and 10 min after exercise the systole in the group of young men was 1.8 ± 1.2 csec (mean ± SD n = 12) longer in relation to heart rate than might be expected from the regression line of the values at rest and during exercise ($P < 0.001$) The absolute values of mechanical systole were however lower than at rest before exercise

Correlation with central circulation

Heart rate at maximal working intensity (HR_{max}) In the group of old men the total and some partial correlation coefficients were studied between the individual slope of mechanical systole on heart rate (the individual regression coefficient) HR_{max}, the 2—6 min heart rate increase at heart rate *130* (St_{130}) and the assessment of the electrocardiographically recorded ectopic beats during the exercise test (Table V) The individual values for the slope of mechanical systole on heart rate were calculated from the linear regression lines for values with heart rates ≤ 130 The only significant correlation was

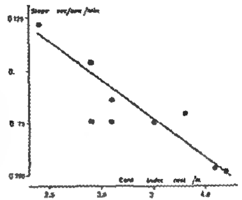

Fig 7 Individual slope (regression coefficient) of mechanical systole on heart rate (y) in relation to cardiac index at rest (x) in supine position in 9 m n aged 61—83 years
$y = -0\,063 - 0\,032x$ SD $= 0\,012$
$r = -0\,87$*

between HR_{max} and St st_{138} By eliminating the influence of St st_{138} and/or the assessment of the ectopic beats during the exercise test the correlation between the slope of mechanical systole on heart rate and HR_{m} was probably significant This indicates that $HR_{m\,x}$ was lowest in cases with the steepest negative slope which would tend to equalize the mechanical systole at maximal working intensity This influence was not very pronounced however as the correlation coefficient between mechanical systole at maximal or close to maximal working intensity and heart rate was as high as — 0 87*** and the regression coefficient (— 0 118) did not differ from the slope of the total regression line (eq 10 Table III) at HR_{ma}

In the young men the total correlation coefficient in supine position between the slope of mechanical systole on heart rate (x) and $HR_{m\,x}$ (y) was only 0 24 After eliminating the influence of the 2—6 min heart rate increase at heart rate 170 (z) the partial correlation coefficient was still of no probable significance (r = 0 21) nor the correlations in sitting position (r = 0 22 r = 0 36) either

Cardiac output Nine of the old men were also studied separately by right heart catheterization in supine position (Granath Jonsson and Strandell 1961)

Table 11 Total and some partial correlation coefficients (r) between the slope of mechanical systole on heart rate in supine position (and residual regression coefficient 1) heart rate at maximal working intensity (2) cardiac index at rest (3) and the highest recorded pulmonary capillary venous pressure during supine exercise (4) in 9 men aged 61—83 years For correlations with parameter 4 n = 8

Total r	Total r	Partual r	Partual r
r_{12} = −0 07	r_{13} = 0.27	$r_{12\,3}$ = 0 6	$r_{12\,34}$ = 0 75
r_{14} = −0..1	r_{23} = −0 87	$r_{13\,2}$ = −0 91	$r_{13\,24}$ = −0 95
r_{34} = 0 18	r_{24} = −0.54	$r_{24\,3}$ = −0 71	$r_{14\,23}$ = −0 76

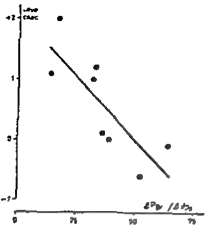

Fig 8 Individual level of mechanical systole on heart rate (y) in relation to the increase in systolic pressure in the brachial artery from rest to exercise per increase of oxygen uptake (x) in supine position in 8 men aged 61–83 years.
$y = 2.1 - 0.043x$ SD = 0.55 $r = -0.81$

In these cases the individual slope of mechanical systole on heart rate (the individual regression coefficient) was found to be correlated to the cardiac output at rest ($r = -0.79^*$) and the arterio-venous oxygen difference at rest ($r = 0.73^*$). The most significant correlation was with the cardiac index at rest ($r = -0.87^{**}$) (Fig 7 Table VI). The further data given in Table VI show that the partial correlation coefficient between slope and cardiac index after eliminating the influence of HR_{max} was highly significant whereas all the partial correlation coefficients with HR_{ms} or the highest recorded pulmonary capillary venous pressure during supine exercise were just below the level of probable significance ($0.1 > P > 0.05$).

For a closer study of the correlation between slope and cardiac index, partial correlation coefficients were computed for the various components of the cardiac index (heart rate, stroke volume and body surface area). None of the total correlation coefficients were of probable significance ($P > 0.1$). After eliminating the influence of the two other factors the correlation coefficients between slope and heart rate was -0.86^*, between slope and stroke volume -0.87^* and between slope and BSA 0.77 ($0.1 > P > 0.05$).

Arterial blood pressure The level of the individual regression lines for mechanical systole on heart rate (≤ 150 beats/min) was correlated to the pressures in the brachial artery in the 9 old men who were also separately studied by right heart catheterization. The level was given as the position of the mean of the line in relation to the regression line for the old men (eq 10 Table III). The correlation coefficients between the level of the line and the systolic pressures at rest were not of probable significance ($0.1 > P > 0.05$). When the level was correlated to the systolic pressure during exercise at heart rate *130 beats/min* the significance increased ($r = 0.74^*$). The correlations with the corresponding mean and diastolic pressures were less significant ($P > 0.2$). The best correlation however was with the increase in systolic pressure from rest to

exercise expressed as mm Hg/l increase in oxygen uptake ($r = -0.81$ $P < 0.02$) (Fig 8)

Some other parameters In the group of old men there was no correlation of probable significance between the slope of mechanical systole on heart rate or the level of the line and age height weight assessment of ST segment ectopic beats conduction disturbances or total changes in the electrocardiogram at rest or during exercise heart volume blood volume W_{170} maximal work load arterial lactate concentration during exercise or anamnestic degree of physical training There was no correlation of probable significance between the highest recorded pulmonary capillary venous pressure during supine exercise and mechanical diastole or systole at maximal working intensity

Discussion

Curvilinearity of mechanical systole on heart rate

The curvilinearity of the regression of mechanical systole on heart rate above 150 beats/min in the young men was due to curvilinearity of the individual curves In the old men no individual curvilinearity could be proved which however may be due to the fewer observations per individual and to the fact that only few subjects were studied above heart rate 150 The curvilinearity in this group seemed to be connected with the probably significant correlation between the slope of mechanical systole on heart rate and the heart rate at maximal working intensity Owing to the different relation between mechanical systole and heart rate during rapidly changing heart rate it is probable that a minor part of the curvilinearity of the individual curves was due to lack of heart rate steady state during the heaviest loads The mean 2—6 min heart rate increase at the heaviest load was 10 beats/min in supine and 7 beats/min in sitting position in the present material of young men For a more thorough study of this curvilinearity therefore both ordinary subjects and well trained athletes who can better maintain a steady state should be investigated

In the present study the curvilinear regression of mechanical systole on heart rate was expressed as a second degree polynomial It could also be obtained by first expressing the mechanical diastole as a hyperbolic function in relation to heart rate (Bevegård 1963) and then computing the systole In the present group of young men the inverted value of mechanical diastole seemed to be linearly related to heart rate and the regression equation for diastole (y csec) obtained in supine position was $y = 10^4/(4.1x - 92)$ SD for $1/y = \pm 2.2x/10^5$ $n = 52$ where x = heart rate in beats/min This line is almost identical to the one given by Bevegård (1963) which would indicate that holding of the breath during the recordings in the present study was of no detectable significance for the regression line The fact that the SD of mechanical diastole was proportional to the length of diastole caused it to be very great

at low heart rates e.g. + 11.1 and — 9.1 csec at heart rate 50 beats/min, + 2.45 and — 1.75 csec at heart rate 100. At heart rate 150 it was + 1.30 and — 1.15 csec. As the standard deviation of mechanical systole calculated as total heart period less diastole will be the same as for diastole this way of predicting mechanical systole in relation to heart rate will be less exact than the second degree polynomial used in the present study, where the standard deviation was ± 1.27 csec at all heart rates studied. The only exception to this is when all the values studied are around or above heart rate 150. Then the standard deviation of mechanical diastole will be lowest.

Variations in slope and level of mechanical systole on heart rate

The slope of mechanical systole on heart rate in the present study was similar to the slope observed at rest for children aged 3—13 years (Mannheimer 1940) and to the slope of the time interval from Q in the ECG to the 2nd sound at rest for male and female medical students (Kuhns 1953). Thus the interindividual variations at rest seem to be similar to the variations during exercise. In atrial fibrillation the ejection time of the left ventricle in relation to heart rate, computed from the duration of the preceding diastole (Blumberger 1958) shows mainly the same slope as mechanical systole in the present study. In atrial fibrillation, however, the decrease in ejection time with decrease in length of diastole is only due to the decreased length of diastole and the decreased ventricular filling and not to an increased sympathetic activity as during exercise. It should be of great interest to study the effect on mechanical systole of varying heart rate in subjects with total AV block and artificial pacemaker.

Effect of body position. The shorter mechanical systole in sitting compared with supine position for a given heart rate is in agreement with the findings of Lombard and Cope (1926). In contrast to the present findings, however, they observed an increasing difference between sitting and supine position at higher heart rates. Probably their method of measuring the ejection time from carotid sphygmograms was less exact than the present phonocardiographic recordings. The shorter systole in sitting position would be connected with the described lower filling pressures and stroke volumes and the increased sympathetic tone in that position (Bevegård, Holmgren and Jonsson 1960). Intraindividual variations in stroke volume accompanied by changes in sympathetic tone among other things are thus connected with changes in the duration of systole wheras interindividual variations in stroke volume are not (see below). Even concerning the intraindividual variations in stroke volume the relationship to variations in duration of systole is not so exact. Thus the differences in duration of systole in sitting compared to supine position at rest and during successively increased work loads did not correspond to the expected differences in stroke volume.

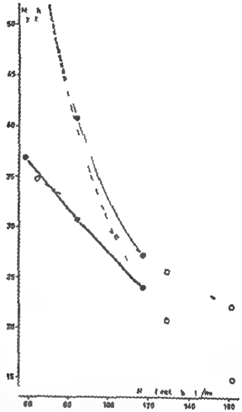

Fig 9 Mechanical systole (heavy lines) and mechanical diastole (thin lines) in relation to heart rate in case no 78 (whole lines) and case no 14 (broken lines)

Effect of age The prolongation with rising age of mechanical systole in relation to heart rate at rest and during exercise was significant but in absolute values rather slight There was no difference in slope and the increase in level in the 71 year-old men compared with the 23 year-old ones was only 0 7 csec This is less than the increase with age of the ejection time observed by Michel (1960) He studied 409 subjects from 8 up to 86 years of age and observed at rest a mean increase of 1 5 csec for the same age interval as in the present study without corresponding changes in heart rate These slight changes with age are observed simultaneous with a decrease in stroke volume at rest of around 30—40 % (Brandfonbrener Landowne and Shock 1955 Granath Jonsson and Strandell 1961) Other factors that may be related to differences in the duration of mechanical systole with rising age are differences in sympathetic nervous activity the higher endsystolic aortic pressures in old age and the increased stiffness in the vascular walls and heart walls with age

The longer mechanical systole in the group of old men at maximal or close to maximal working intensity was thus mostly connected with their lower heart rates This indicates that the major fraction of the differences in maximal heart

rate between young and old men cannot directly be explained by changes of the systolic or diastolic time intervals

Correlation with heart rate at maximal working intensity The slight correlation observed within the group of old men between the slope of mechanical systole on heart rate and the heart rate at maximal working intensity (HR_{max}) is interesting, as there was no difference in the mean slope between the young and old men although the HR_{max} was 38 beats/min lower in the old

The effect of different slopes on the duration of systole and diastole can be illustrated by two extreme cases, no 14 having a flat slope and a high HR_{max} and no 78 having a steep slope and a low HR_{max} (Table II, Fig 9) At 70 beats/min the two men had the same mechanical systole and diastole When case no 78 broke the test at heart rate 121 his mechanical systole was still *shorter than the diastole and approximately 3 5 csec shorter than the systole* of case no 14 At this heart rate case no 14 had a diastole that was shorter than the systole When case no 14 broke the test at 162 his mechanical systole was 2 csec shorter than the value for case no 78 at heart 117 and his diastole was markedly shorter than the systole (7 csec) Marked interindividual differences in the duration of systole and diastole during exercise were also observed by Holmgren (1956) and the present findings may raise some questions Was the low heart rate during submaximal and maximal exercise and the shorter systole in case no 78 an adaptation in order to maintain a sufficiently long filling time for the heart? Or had case no 78 primarily a less pronounced chronotropic sympathetic effect on the heart during exercise than case no 14?

Correlation with cardiac output The significant correlation observed between the cardiac index at rest and the slope of mechanical systole on heart rate is *probably not a direct one but is ascribable to both factors being influenced* by the sympathetic and parasympathetic nervous activity If this was the case the correlation mentioned above may be explained by the hypothesis that the subjects with the steep slope had a decreased vagal activity at rest giving a high heart rate and an increased inotropic sympathetic stimulation with consequent increase in stroke volume Their steep slope and (in the total material) probably significantly lower HR_{max} may be explained by a lesser chronotropic stimulation or response than for the subjects with the flatter slopes and higher HR_{max}

Correlation with arterial pressure The significantly decreased level of mechanical systole in relation to heart rate in the cases with *most marked increase* of systolic pressure during exercise agrees with the findings of Wiggers (1921) who observed a shortening of systole at moderately increased arterial resistance *and increased aortic pressures* An increased inotropic sympathetic effect would both lower the level and give a higher increase of the systolic arterial pressure during exercise

Effect of some other parameters Neither the level nor the slope of the regression line of mechanical systole on heart rate was primarily *affected to a probably*

significant degree by interindividual variations in heart volume physical working capacity stroke volume blood volume or electrocardiographic changes This is in agreement with the findings of Jonsson (1958) who observed the same duration of mechanical systole of the right ventricle in normal subjects and in subjects with atrial septal defects with stroke volumes up to 4 times the normal Nor were differences observed in normal subjects differing in physical working capacity and stroke volume by Garlind et al (1960) However in the present study the correlation between the slope of mechanical systole on heart and the stroke volume at rest was probably significant after eliminating the influence of heart rate and body surface area

Effect of rapidly changing heart rate After 1 min of exercise at the different loads the mechanical systole was significantly longer in relation to heart rate than after 5 min During the first 3—4 min after exercise the mechanical systole was shorter in relation to heart rate than during exercise This indicates that the changes in systole were slower than the changes in diastole both on increasing and decreasing of the heart rates Similar findings were observed by Katz (1921) on anesthetized dogs and by Blair Wedd and Young (1941) on isolated turtle heart strips The latter authors also noted that the Q—T interval in the ECG in human subjects was shorter after exercise in relation to heart rate and that later the Q—T interval continued to lengthen until it even became supernormal

The measurement of the Q—T interval in the ECG after exercise has been used to differentiate between normal subjects and subjects with cardiovascular diseases (Yu *et al* 1950) The abnormal response would consist of absence of shortening of the corrected Q—T interval during the first min after exercise The corrected Q—T interval (Q—T corrected $= \dfrac{\text{observed Q—T interval}}{\sqrt{\text{cycle length}}}$) however increases with increasing heart rate during exercise in normal subjects (Yu et al 1950) An absence of shortening after exercise may thus be due solely to a persistent high heart rate after exercise and not necessarily to changes in mechanical systole in relation to heart rate In the present material the mechanical systole shortened after exercise in the same way in the old and young men Within the group of old men those who had abnormal or suspected abnormal electrocardiographic findings during the exercise test did not differ from the remainder

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Noradrenaline and Acetylcholinesterase in Sympathetic Ganglion Cells of the Rat

By

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In a previous histochemical study it was shown that the noradrenaline (NA) concentration of individual nerve cells of the superior cervical ganglion of the rat ranges from low to high through intermediate stages (Eranko and Harkonen 1963) In the present report the distribution of NA is compared with that of acetylcholinesterase (AChE) activity

A method was employed which allows demonstration of NA and AChE in the same section (Eranko 1964) Frozen dried superior cervical ganglia of the rat were exposed to dry formaldehyde vapour at 50—55 C for 1 hour which makes NA fluorescent but does not destroy AChE After fluorescence photomicrography AChE activity was demonstrated with Gomori s (1952) method using acetylthiocholine as a substrate and 10^{-4} M tetra isopropylpyrophosphoramide to inhibit non specific cholinesterase

Fig 1 shows formaldehyde induced fluorescence due to NA Fig 2 is of the same section after demonstration of AChE Examination of such pairs of photomicrographs proved that weak moderate or strong fluorescence may be associated with weak moderate or strong AChE activity Many strongly fluorescent cells exhibited a marked AChE reaction

If it is accepted that nerve cells with much NA are adrenergic and those with an intense AChE activity cholinergic (see Koelle 1962) our results suggest that some sympathetic ganglion cells may be both adrenergic and cholinergic at the same time This is of special interest in view of pharmacological observations apparently indicating that stimulation of a cholinergic sympathetic fiber causes a liberation of acetylcholine which in turn may exert its effect by liberating NA from the same fiber or an adjacent one (Burn and Rand 1962) Our preliminary studies have indeed suggested that some nerve fibers of the rat iris may contain both NA and AChE

Hamberger Norberg and Sjoqvist (1963) compared the distributions of NA and AChE in neighbouring sections of cat s sympathetic ganglia. In contrast to our observations they never found monoamines and significant levels of AChE in the same neuron The discrepancy between their and our observations may well be due to species differences (see Burn and Rand 1962 Koelle 1962)

This study has been supported by grants from the Sigrid Juselius Foundation and from the National Institute of Arthritis and Metabolic Diseases U S Public Health Service (A 1725)

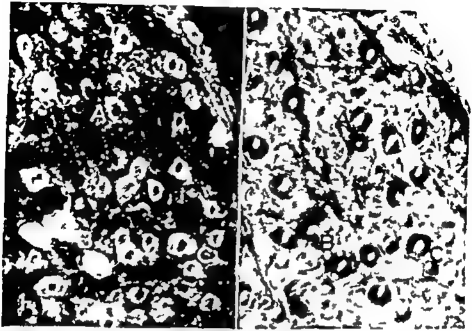

Fig 1 Formaldehyde induced fluorescence in the superior cervical ganglion of the rat showing the distribution of noradrenaline

Fig 2 The same section after demonstration of acetylcholinesterase activity Three corresponding sites in both figures are indicated with letters to the left of which are A two weakly fluorescent cells with a weak acetylcholinesterase activity B two strongly fluorescent cell with a strong acetylcholinesterase activity C two moderately fluorescent cells with a strong acetylcholinesterase activity

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The Origin of Blood withdrawn from Deep Forearm Veins during Rhythmic Exercise

By

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Abstract

IDBOHRN H and J WAHREN *The origin of blood withdrawn from deep forearm veins during rhythmic exercise* Acta physiol scand 1964 *61* 301—313 — Healthy subjects were studied during rhythmic forearm exercise in respect of variations in oxygen saturation in blood samples from deep and superficial forearm veins at ordinary room temperature and during body heating (Series A) The oxygen saturation in blood from deep veins was not changed by variations in the oxygen saturation of superficial venous blood The elevation of pressure in deep veins during contractions was significantly greater than in the superficial venous system After the injection of contrast medium and dye in the radial artery during work (Series B and C) neither of these indicators could be found in significant amounts in the deep veins when the intensity of work exceeded 4 kpm/min at contraction frequencies of 60 and 90 min and 5 kpm/min at 30 contractions per min. The study shows that superficial venous blood from the hand or forearm does not mix to a significant extent with the deep venous blood during rhythmic work of this type and of a certain minimum intensity

The possibilities of studying human muscle metabolism and circulation in vivo have been limited by the difficulty of obtaining pure muscle blood The forearm however offers possibilities in this respect As was shown by Holling (1939) Mottram (1955) and Coles *et al* (1958) blood draining the forearm muscles almost exclusively can be obtained at rest after arterial occlusion at the wrist via a catheter inserted percutaneously into a deep forearm vein It seems moreover that such blood can also be obtained from the deep veins without wrist occlusion during rhythmic exercise with a hand ergometer (Pernow and Wahren 1962) Dye infused into the radial artery in conjunction with exercise (work intensity 7 5 kpm min) could not be demonstrated in the deep veins

There are two systems of veins in the forearm one deep and one superficial The deep veins consist of *venae comitantes* to the arteries of the forearm Of

these, the radial and ulnar veins communicate with the veins of the hand while the interosseous only drains the deep structures of the forearm. At the wrist the deep and superficial veins connect via thin valveless anastomoses (Lavarzzario and Ottolini 1958). In the proximal part of the forearm the deep veins form a vascular network which communicates through the deep median cubital vein with the basilic or the cephalic vein. The deep venous system unites at the bend of the elbow to form the brachial venae comitantes. Opinions differ as to whether the antebrachial fascia is penetrated by veins elsewhere than in the vicinity of the joints. No large veins pass through the fascia (Lavarzzario and Ottolini 1958) but the existence of small functionally important communications cannot be excluded (Rauber Kopsch 1955).

The object of the present study was to throw further light on whether blood from the hand or the skin of the forearm occurs to any significant degree in the deep veins during work. This question has been studied both by the analysis of variations in oxygen saturation in deep and superficial veins in the forearm during work at varying skin blood flow (series A) and by the administration of dye and contrast medium in the radial artery during work (series B and C).

Material

The material consisted of 27 healthy male subjects 17—41 years old (mean = [illegible]). Twelve were firemen, five medical students and the remainder were patients who before the investigation had been treated for minor surgical afflictions of no importance for the present investigation.

Procedure

Teflon catheters were introduced percutaneously into a superficial and a deep vein of the right forearm and in some cases also in the radial artery. The deep venous system could normally be reached via the median cubital vein but in [illegible] cases the cephalic vein was used. By manipulating the catheter, if necessary in conjunction with venous stasis, it was always possible to pass the catheter upstream so far in that the tip could not be palpated. The catheter had then been introduced at least [illegible] cm. The radial artery was punctured at the level of the radial styloid process after the subject had kept his arm in hot water for about 10 minutes. This catheter was introduced downstream [illegible] cm.

Work was performed in the supine position with a hand ergometer in time with a variable electric metronome. The work consisted of compressing two spring-loaded handles in the hand (path of contraction [illegible] mm). The distance between the two handles was adjusted before the beginning of the experiment and then not changed. Work per contraction could be varied by altering the pre-set tension of two different calibrated springs.

Series A. The subjects arrived at the laboratory in the morning after a light breakfast. They were coolly clothed during the experiment. The room temperature varied during the first part of the experiment between [illegible] and [illegible] °C. When catheters had been introduced into a deep and a superficial vein, resting values for oxygen saturation were recorded with free hand circulation and with arterial occlusion at the wrist. Thereupon rhythmic exercise was performed for 15 min ([illegible] kpm/min, [illegible] contractions per min). Blood samples were taken repeatedly during exercise for analysis of oxygen saturation. After this a

heat cupboard with a fan was placed over the thorax and the subject was warmed up for 30—40 min with 40 C air The previous experimental procedure was then repeated during continued heating

Series B The serum creatinine concentration was checked in all subjects in this series at the outset to disclose renal insufficiency When catheters had been introduced into a deep and a superficial vein and into the radial artery 8 of 10 subjects were given 5 ml Baralgin© (Hoechst) i v to prevent vasospasm during the course of the experiment The contrast medium (4—10 ml 45 per cent Urografin/sodium and methylglucamine salts of diatrizoate Schering®) was injected into the radial artery at an even rate by hand over a period of 3—10 sec. Beginning 3—7 5 sec after the start of injection 10 films were exposed at a rate of 1 film per sec The exposures were made in 2 planes (frontal and side) at rest and during rhythmic work (4 4 kpm/min 60 contractions per min) During exercise the injection was made 3—4 min after the start of the work period The vein catheters were filled with contrast immediately before the exposures to facilitate identification on the films

In 9 cases after the x ray investigation 0 01 per cent indocyanine green solution (Cardio-green) was *infused at a constant rate (3 ml/min) into the radial artery* Dublicate blood samples were taken from superficial and deep forearm veins at rest and 3—4 min after the beginning of exercise

Series C After introduction of catheters into a deep and a superficial vein and into the radial artery indocyanine green solution was infused in 7 cases at a constant rate in the radial artery in connection with work of varying intensity Infusion of dye sampling and analysis were carried out as in series B The intensity of work was varied partly by work per contraction being changed by altering the spring tension and partly by work being carried out with different frequencies of contraction The frequencies used were 30 60 and 90 contractions per min with 0 5—5 0 kpm/min at the first and 1—4 kpm/min at the other frequencies

Methods

Oxygen saturation was determined spectrophotometrically with a slight modification of Drabkin s (1950) technique The error of analysis for oxygen saturation values expressed as the coefficient of variation for one determination was 4 1 per cent in the range 0—33 per cent saturation 1 8 per cent in the range 34—67 per cent and 0 6 in the 67—100 per cent range These values have been calculated on the basis of double analyses of the same blood sample The coefficient of variation calculated from the analysis of two samples obtained at rest with an interval of no more than 5 min was 5 4 per cent (n 18 mean 60 1 per cent oxygen saturation)

The concentration of indocyanine green in plasma was measured spectrophotometrically at the point of maximal absorption close to 805 mμ Correction was made for recirculating dye The standard deviation for a single analysis of dye concentration in the range 0—7 mg/l was 0 10 mg/l This value was calculated from 20 sets of simultaneously obtained duplicate blood samples When the concentration of dye in venous samples during work exceeded the arterial concentration by less than 0 32 mg l the difference was considered insignificant (confidence 99 per cent) The tolerated difference constitutes 2 6 per cent of the mean dye concentration of blood samples from deep veins during infusion of dye at rest in series B and C (12 1 mg/l)

Skin temperature measurements were made with a *thermocouple and a direct reading* galvanometer (TE 3 Ellab)

Venous pressure was recorded with an electromanometer (Elema Schonander) connected to an ink jet recorder (Mingograph 42 B Elema Schonander) The reference point for zero pressure was taken as the mid thoracic point at the level of the insertion of the fourth rib

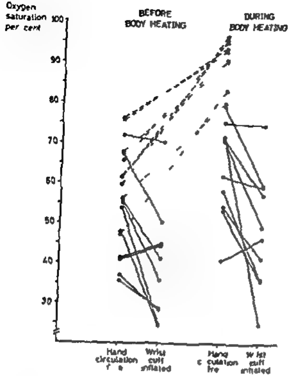

Fig 1 Oxygen saturation values recorded at rest in blood samples from deep (———) and superficial veins ( ) before and during body heating with and without wrist occlusion

Results

Series A

Rest This series covers 10 exps The oxygen saturation for blood samples from superficial and deep veins at rest with and without arterial stasis at the wrist both before and during heating is given in Fig 1 Oxygen *saturation values in blood samples from deep veins after wrist occlusion were* significantly lower ($p < 0.001$) than corresponding values in blood from superficial veins both before and during body heating In the great majority of cases the oxygen saturation of the blood from deep forearm veins fell after wrist occlusion *In 3 cases however no great difference in this respect was noted* with and without stasis There is therefore reason to suppose that in the latter cases the deep vein in which the tip of the catheter lay did not drain blood from the hand The oxygen saturation of blood samples from deep veins with and without wrist occlusion are probably significantly higher (p 0 05) during heating than before

Work

Deep venous blood Fig 2 shows the variations in oxygen saturation measured before and during heating in blood samples from deep and superficial forearm veins during work (15 min The oxygen saturation in deep venous blood was

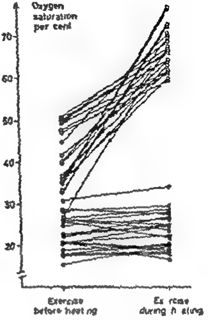

Fig. 2. Oxygen saturation values recorded in 10 experiments during exercise before and during body heating in blood samples from superficial veins (——•) and deep veins (——)

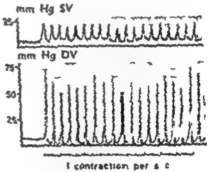

Fig. 3. Pressure recordings from a deep (DV) and a superficial (SV) forearm vein at work intensity 4.4 kpm/min and 60 contractions per min. The recordings were made immediately following each other.

22.5 ± 3.9 per cent (M ± S.D.) during the first work period and 23.9 ± 4.3 per cent during body heating. The difference is not significant ($p > 0.1$). The oxygen saturation values of blood samples obtained after 10 and 15 min do not differ significantly. The coefficient of variation for a single observation during work calculated from paired 10 and 15 min samples between the two work periods was 11.7 per cent ($n = 10$, mean 23.2 per cent oxygen saturation).

Superficial venous blood. The oxygen saturation values for superficial venous blood were 40.5 ± 7.3 per cent before heating and 65.4 ± 5.8 per cent during heating. The difference is significant ($p < 0.001$). After the transition from rest to work the oxygen saturation in superficial venous blood had decreased by

a b

Fig 4 (a) Reflux of contrast from the radial artery to the whole arterial system of the forearm following injection into the radial artery at rest The two venous catheters are visualized by contrast filling and the tips indicated by arrows
(b) Delayed filling of the veins in the forearm Remnants of contrast medium are still seen in the arteries 6 sec after start of injection Same injection as in (a)

22 5 ± 8 4 per cent in the experiments performed at ordinary room temperature The corresponding value for the experiments during body heating was 24 5 ± 8 2 per cent The difference between these two values is not significant (p 0 1)

The skin temperature during work was recorded in 4 cases from the middle of the volar surface of the forearm the back of the hand and the palm The mean increase in skin temperature before and during heating was on the forearm 5 4 ((3 5—7 7) on the back of the hand 7 8 (6 1—9 2) and on the palm 8 3 (6 8—12 4)

The pressure variations in superficial and deep veins in connection with work (4 4 kpm min 60 contractions per min) were determined in 6 cases In order to secure a free position of the tip of the catheter blood sampling always preceded the pressure recordings The pressure at rest was 4 7 ± 1 7 mm Hg in deep veins and 3 7 ± 2 7 mm Hg in superficial veins The difference is not significant During work the pressure increased during contractions by 51 9 ± 21 6 mm Hg over the resting level in deep veins and by 8 3 ± 4 2 mm Hg in superficial veins (Fig 3) The difference in pressure increase during contractions is significant (p < 0 01)

Table I Findings of contrast medium and dye after administration in the radial artery at rest and during work (4 4 kpm/min 60 contractions per min) in 10 subjects

	At rest					During exercise				
	Presence of contrast medium in			Presence of infused dye in		Presence of contrast medium in			Presence of infused dye in	
Subjects	Catheter indicated DV	Other DV	Catheter indicated SV	DV	SV	Catheter indicated DV	Other DV	Catheter indicated SV	DV	SV
TI	−	−	−	−	+	−	−	+	−	+
PO	−	−	1)	+	+	−	−	1)	−	+
PR	−	−	+			−	−	+		
LL	+		+	+	+	−	−	+	−	+
SC	−	−	−	+	+	−	−	+	−	+
SB	+		+	+	+	−	−	+	−	+
TJ	−	−	−	−	2)	−	−	−	−	2)
EC	−	−	+	+	+	−	−	+	−	+
RN	−	+	+	+	+	3)	−	+	−	+
VP	−	−	−	−	+	−	−	−	−	+

1) Catheter not filled with contrast medium
2) No sample obtained due to vasospasm.
3) Presence of contrast in deep vein doubtful in one series definitely negative in repeated experiments

Series B

This series covers 10 exps The contrast medium gave satisfactory filling of the forearm veins and permitted in all cases differentiation between superficial and deep veins The films were studied for the presence of contrast medium both in the catheterized superficial and deep veins and in other veins The injection of contrast medium at rest usually elicited transitory pain in the hand and sometimes also in the lower forearm The intensity of the pain varied from individual to individual but was throughout most intense in some cases when there occurred a reflux of the contrast to the forearm arteries (Fig 4) The appearance of contrast in the forearm veins was in these case delayed Repeated slower injection of contrast about 15 min later combined if necessary with manual compression of the radial and ulnar arteries immediately proximal to the wrist gave no significant reflux During work there was no reflux in any case and the pain on injection was reported to be mild The veins always filled well and considerably more rapidly than at rest

At rest The position of the catheter in the deep vein could be confirmed in all cases The results of the x ray investigation and the dye infusion are given in

a b

Fig 5 (a) Following injection into the radial artery at rest contrast medium is seen in both deep and superficial veins The two venous catheters are visualized by contrast filling and the tips indicated by arrows (b) No contrast can be seen in the deep veins after injection during exercise (4 4 kpm min 60 contractions per min Exposure was made 6 sec after start of injection

Table I At rest deep veins filled in 3 cases 2 of which were the catheterized deep vein In these 3 and a further 3 cases there was dye in the blood sample from the catheterized deep vein A large number of superficial veins always filled including the catheterized vein in 5 cases In one case the superficial catheter could not be located owing to contrast leakage and in one case spasm of the catheterized superficial vein made it impossible to obtain blood samples In all cases where samples were taken there was dye in the blood from the superficial veins

Work As a rule no contrast could be observed in the deep veins Fig 5 In *one case however a minute amount of contrast medium appeared in the catheterized* deep vein during work in one series Repeated injection did not cause this vein to fill again In all 9 cases examined blood samples from the deep veins during dye infusion were free from significant concentrations of dye

Series C

The concentration of indocyanine-green in blood samples from deep forearm veins after the infusion of dye in the radial artery during work of varying intensity is shown in Fig 6 The concentration declined with increasing intensity of

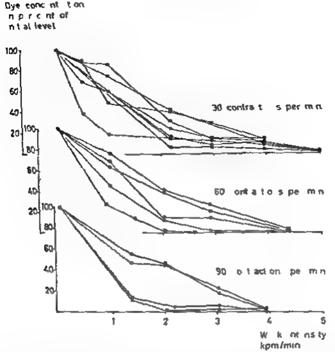

Fig 6 Concentration of dye in deep venous blood during exercise of varying intens ty The dye was continuously infused into the radial artery at rest and during work The concentration during exe e is exp essed as a percentage of the concentration ecorded during infusion at rest

work and no dye appeared in significant concentrations at work intensities of approximately 4 kpm/min or more at contraction frequencies of 60 and 90 per min In work at a frequency of 30 contractions per min and 4 kpm/min there was a low but significant concentration of dye in the deep venous blood but at 5 kpm/min no dye could be detected

The dye concentration in blood from superficial veins showed irregular variations Usually the concentration decreased during work though it did rise in some cases 30 per cent of the rest value was the lowest concentration measured during work

Discussion

Series 4

In connection with body heating the blood flow of the hand increases (Lewis and Pickering 1931 Gibbon and Landis 1932) as does the skin blood flow in the forearm (Roddie Shepherd and Whelan 1956 Edholm Fox and Macpherson 1956) while muscle blood flow appears to remain unchanged (McGirr 1952 Barcroft *et al* 1955) The increase in the blood flow of the hand is prob-

ably caused by *release of vasoconstrictor tone* Apart from this mechanism there may also be an active vasodilation in the forearm skin in connection with body heating (Roddie *et al* 1957)

At rest the oxygen saturation measurements of deep venous blood tended to be higher during heating than before In the case of observations made with a free hand circulation this is probably due in part to an increased oxygen saturation in the component of venous blood from the hand The same tendency was observed however in blood samples obtained during wrist occlusion It may then be due rather to the fact that the body heating was preceeded by a work period than to the heating itself As indicated by Pernow and Wahren (1962) the oxygen saturation of deep venous blood may still exceed the resting value 45 min after the end of work In two cases the period of rest after the first exercise was prolonged and the total time between work periods was one hour The differences between the second and the first values in these two cases were — 1 0 and + 2 2 per cent oxygen saturation

In 3 of the subjects studied practically unchanged oxygen saturation values were recorded at rest before and during wrist occlusion (Fig 1) This finding indicates that the tip of the deep venous catheters probably lay in a vein of the type *v interossea* The results obtained in the work experiments in these cases did not differ from the rest of the group

During work comparable levels of oxygen saturation were recorded in deep venous blood at an ordinary temperature and during body heating This probably indicates that the superficial venous blood does not contaminate the deep venous system during this type of work Certain conditions must be fulfilled however for this statement to be valid The arterial oxygen saturation is presumed to remain unchanged This has been shown to be the case both during exercise (*Holmgren and Linderholm 1958 Pernow and Wahren 1962*) and during body heating (Roddie *et al* 1956) It is also presupposed that muscle metabolism and muscle blood flow are the same during both work periods At rest forearm muscle metabolism (Roddie *et al* 1956) and blood flow (McCirr 1952 Barcroft *et al* 1955) appears to remain unchanged with body heating In order to illustrate that this is probably also the case during exercise the temperature in the *flexor digitorum profundus* was recorded during work with a thermocouple mounted in an injection needle in one experiment The temperature during exercise was the same within a tenth of a centigrade before and during body heating

The evidence that the superficial venous blood does not pass to the deep veins is further substantiated by the finding of considerable pressure elevations during exercise in the deep veins This is probably due to the deep veins bein compressed by the muscles during contractions The pressure gradient between the deep and superficial veins during work suggests that the flow of blood in the connections between the two venous systems is from the deep towards the superficial veins

The oxygen saturation in the superficial venous blood fell markedly at the transition from rest to work and it decreased by the same amount with and without body heating This series of experiments cannot throw any further light on the question whether this is due to admixture of blood from deep veins increased vasoconstrictor tone in the hand and the skin of the forearm or decreased vasodilator tone in the forearm skin

Series B

Eight of ten subjects in this series were given 5 ml of the spasmolytic and analgetic agent Baralgin© before the experiment in an attempt to prevent vasospasm and pain at the injection of contrast medium No record of circulatory effects of this substance has been found It seems likely however that this effect if any would be to facilitate blood flow and open up communications between the deep and superficial venous systems rather than the inverse

Most authors report unchanged or increased blood flow after intraarterial injection of contrast medium (Sako 1963) both during cerebral angiography (Kagstrom Lindgren and Tornell 1958 and 1960) and renal angiography (Idbohm and Nordgren to be published) Shaw (1956) however observed immediate cessation or reduction in amplitude of toe pulsations in most cases following injection of the contrast media Diodrast or Urokon into the femoral artery After one to six minutes an increase in amplitude to or above the original level was registered This finding is considered a result of a transient arterial vasoconstriction due to a direct effect of the contrast media on the arteries

It seems that in the present series the hand arteries were similarly sensitive to the contrast medium In 3 cases frontal pictures of the hand were taken in conjunction with contrast injection The pictures show filling of both the superficial and deep carpal arches and the digital arteries When the injection was painful incomplete filling of the digital and other small arteries was observed It seems possible that the disagreement between the findings of contrast and dye in the deep veins at rest (Table I) can be ascribed to a higher vascular tone during and immediately after the contrast injection The high peak concentration of contrast at the moment of injection into the radial or femoral arteries as compared to the result of injections into the renal or internal carotid arteries may in part explain the varying reports of circulatory effects of contrast media

During work significant amounts of contrast could not be detected in the deep veins of the forearm in any case except one although the superficial veins always were clearly visible The exception was moreover a doubtful case and no contrast was found in the deep vein after renewed injection At rest on the other hand the study was positive as regards both contrast and dye A possible explanation is that this subject had not carried out work satisfactorily during the first contrast injection

There is good agreement between the observations of contrast and dye in the deep veins during exercise The result of the experiments in this series thus con

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Acta physiol scand 1964 61 314—322

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Glycogen Content of Rat Diaphragm after Intraperitoneal Injection of Insulin and Other Hormones

By

Ole J Rafaelsen

Received 6 December 1963

Abstract

Rafaelsen O J *Glycogen content of rat diaphragm after intraperitoneal injection of insulin and other hormones* Acta physiol scand 1964 *61* 314—322 — Intraperitoneal injection of insulin 100 μU per 100 g weight to intact rats causes a 25 to 50 % increase in diaphragm glycogen 10 000 μU insulin causes a 200 to 400 % increase in diaphragm glycogen These insulin doses do not influence blood glucose levels Intravenous injection of insulin 10 000 μU per 100 g weight to intact rats causes a 25 % increase in diaphragm glycogen and in heart glycogen A significant fall in blood glucose is always seen I p injection of growth hormone glucagon desoxycorticosterone and acetylsalicylic acid cause an increase in diaphragm glycogen On a weight basis — and on the basis of physiological effect — the doses needed to increase diaphragm glycogen are far greater than those of insulin The hypothesis is advanced that insulin injected i p in a dose of 10 000 μU or less per 100 g weight to *intact rats becomes bound to the serosal linings of the peritoneal cavity the* hormone exerts only an effect on tissues lining the peritoneal cavity Studies using this system are thus *in vivo* and yet provide some degree of isolation of these tissues

Isolated tissue preparations have been used to study many aspects of intermediary metabolism These preparations have certain advantages conditions *may be changed more radically than in the living organism* measurements may be refined and the possibility of counter action from the organism as a whole is eliminated

It is evident that results obtained under the artifical conditions which prevail in *in vitro* experiments with isolated tissue samples only demonstrate that certain metabolic events may take place under certain circumstances while they do not give any certainty that these same events take place in the intact organism The information obtained may be both quantitatively and qualitatively misleading Results obtained with simplified systems need always be

qualified by their demonstration in more complex and intact systems While it is often difficult to plan and conduct such experiments in the intact organism these remains essential in order to avoid uncritical superstructurization based on test tube results

This paper deals with the effect of insulin and some other hormones on glycogen content of diaphragm (and other muscle samples) in the intact living rat after intraperitoneal injection These studies resulted from the chance observation that insulin administered i p in doses too small to cause systemic effects exerts a profound influence on glycogen content of the diaphragm thereby suggesting that some of the results obtained by workers in many laboratories with the rat diaphragm *in vitro* might be retested *in vivo*

Experimental

Animals Male and female albino rats (Wistar strain) weighing 80 to 120 g were used The animals were fasted for 18 hours (water ad libitum) In any one experiment only rats of the same sex were used though there was no apparent difference between results obtained with male and with female rats

Anesthesia Diaphragms and other samples were isolated from animals anesthetized by inhalation of a mixture of 50 % carbon dioxide and 50 % oxygen for 2 min

Blood glucose In all experiments blood samples were drawn for determination of glucose

Procedure After an 18 hours fast the animals received an injection of 1 ml of saline without or with insulin (or other hormone) In most experiments the i p route was used and the injection made with a morphine cannula (0 60 × 26 mm 24 0.5 gauge short beveled) to the right of the umbilicus In some experiments the 1 ml injection was administered into the femoral vein Sixty to 180 min later the animals were anesthetized and the diaphragms and other muscle samples were excised The diaphragm was studied in all experiments Other samples included heart muscle hind limb muscle and epididymal fat Blood samples were drawn from the neck vessels The diaphragms and other samples were dissected on a block of ice and immediately placed in 0 5 ml 30 % potassium hydroxide

In each experiment 8 to 24 rats were used divided into groups of 4 The control group was always injected with 1 ml 0 9 % saline Other groups were injected with saline containing glucagon free insulin (27 U/mg) in amounts varying from 100 to 10 000 μU per 100 g rat weight in the i p experiments from 1 000 μU to 10 U in the i v experiments Human and bovine growth hormone was administered in doses from 10 to 1 000 μg per 100 g rat weight glucagon in doses from 1 to 1 000 μg desoxycorticosterone hemisuccinate in doses from 10 to 100 μg adrenaline in doses from 1 to 10 μg acetylsalicylic acid in doses from 10 to 1 000 μg per 100 g rat weight

Analytical methods **Glycogen content** The tissue samples were digested in 0 5 ml boiling 30 % KOH and precipitated according to LeBaron (1955 Glycogen was hydrolyzed by adding 2 ml 0 6 N hydrochloric acid followed by boiling for 2 1 2 hours After adjusting the pH to 7 an aliquot was estimated by the glucose oxidase method using the Boehringer reagents Glycogen recovery was between 90 and 95 % Glycogen content was expressed as mg glycogen/g of wet tissue sample

Blood glucose Blood samples were deproteinized with 10 % trichloracetic acid and glucose determined in the supernatant by the glucose oxidase method using the Boehringer reagents The results were calculated as mg of glucose 100 ml blood In ex

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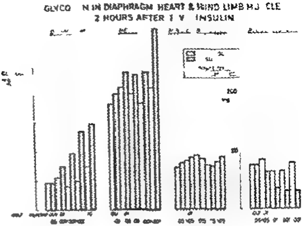

Fig 3 Glycogen content of rat diaphragm 2 hours after intra-arterial injection of 1 ml saline without or with insulin in doses from 0 001 to 10 U per 100 g weight to intact overnight fasted rats

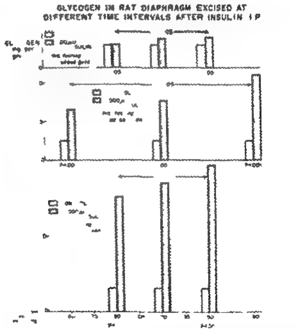

Fig 4 Glycogen content of rat diaphragm 60 to 180 min after intraperitoneal injection of 1 ml saline without or with insulin in doses from 100 to 10 000 μU per 100 g weight to intact overnight fasted rats

fall in blood glucose after i p injection of 10 000 μU insulin never reached statistical validity Fig 2 shows that even 100 μU insulin per 100 g rat weight administered by the i p route increases diaphragm glycogen after 2 hours The dose response relationship in the range from 100 to 10 000 μU is also seen

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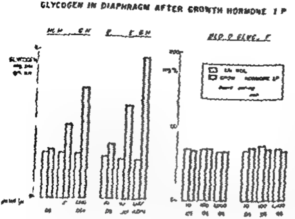

Fig 5 Glycogen content of rat diaphragm 2 hours after intra peritoneal injection of 1 ml sa line without or with growth hormone in doses from 10 to 1 000 μg per 100 g weight to intact overnight fasted rats

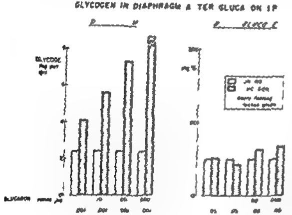

Fig 6 Glycogen content of rat diaphragm 2 hours after intra peritoneal injection of 1 ml sa line without or with glucagon in doses from 1 to 1 000 μg per 100 g weight to intact over night fasted rats

in this figure as is the lack of effect of insulin on glycogen content of epididymal fat

Fig 3 shows that 0 01 U (i e 10 000 μU) insulin per 100 g rat weight is about the smallest dose which increases diaphragm glycogen after i v administration Even greater doses of insulin do not lead to an increase of diaphragm glycogen comparable to that obtained by 10 000 μU insulin via the i p route The parallelism between the insulin effect on glycogen of diaphragm and heart after i v injection is also seen in contrast to the findings after i p injection In all experiments with i v injection of 10 000 μU insulin per 100 g rat weight the fall in blood glucose was statistically significant again in contrast to the findings after i p administration

Prolongation of the interval from i p injection to sampling of tissues leads to a greater insulin effect on diaphragm glycogen (Fig 4) This was observed at 3 different dose levels from 100 to 10 000 μU insulin per 100 g rat weight

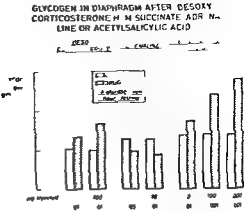

Fig. 7. Glycogen content of rat diaphragm 2 hours after intraperitoneal injection of 1 ml saline without or with desoxycorticosterone hemisuccinate in doses of 10 or 100 μg, adrenaline 1 or 10 μg or acetylsalicylic acid 10, 100 or 1 000 μg per 100 g weight to intact overnight fasted rats.

It was next investigated whether other substances, especially hormones, were able to increase diaphragm glycogen after their i.p. injection. Growth hormone and glucagon administered i.p. both produce remarkable increases in diaphragm glycogen (Fig. 5 and 6), although the doses injected were considerable. Preparations of both bovine and human growth hormone are effective: on a weight basis bovine growth hormone is the more active. Whereas growth hormone in the doses administered has no effect on blood glucose values, glucagon in doses of 100 and 1 000 μg per 100 g rat weight increases blood glucose to some extent.

Desoxycorticosterone hemisuccinate and acetylsalicylic acid increase diaphragm glycogen after i.p. injection (Fig. 7), adrenaline reduces it. The last mentioned substances have no influence on blood glucose values in the doses applied.

Discussion

The results reported illustrate the paramount importance of the route of administration of insulin. This has been stressed by Madison and Unger (1958) in their work describing a direct action of insulin on the liver. The change in response of different tissues as a result of a change of route of administration is closely related to the problem of binding of insulin either to tissues or to blood constituents. Binding of insulin to a tissue was first clearly demonstrated by Stadie and his group (1949 a and b, 1952). Dipping of isolated rat hemidiaphragm in insulin containing solution for 10 sec. led to attachment of insulin to the tissue and several washes in buffer were unable to remove the insulin, as demonstrated by increased glucose uptake by the hemidiaphragm during subsequent incubation. This finding has been confirmed by other workers and extended to fat tissue in experiments with adipose tissue (Haugaard and Marsh

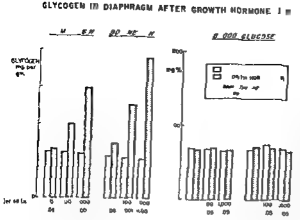

Fig 5 Glycogen content of rat diaphragm 2 hours after intraperitoneal injection of 1 ml saline without or with growth hormone in doses from 10 to 1 000 μg per 100 g weight to intact overnight fasted rats

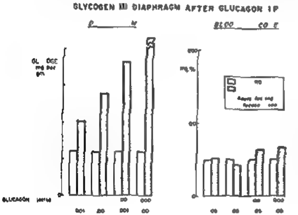

Fig 6 Glycogen content of rat diaphragm 2 hours after intraperitoneal injection of 1 ml saline without or with glucagon in doses from 1 to 1 000 μg per 100 g weight to intact overnight fasted rats

on this figure as is the lack of effect of insulin on glycogen content of epididymal fat

Fig 3 shows that 0 01 U (i e 10 000 μU) insulin per 100 g rat weight is about the smallest dose which increases diaphragm glycogen after i v administration Even greater doses of insulin do not lead to an increase of diaphragm glycogen comparable to that obtained by 10 000 μU insulin via the i p route The parallelism between the insulin effect on glycogen of diaphragm and heart after i v injection is also seen in contrast to the findings after i p injection In all experiments with i v injection of 10 000 μU insulin per 100 g rat weight the fall in blood glucose was statistically significant again in contrast to the findings after i p administration

Prolongation of the interval from i p injection to sampling of tissues leads to a greater insulin effect on diaphragm glycogen (Fig 4) This was observed at 3 different dose levels from 100 to 10 000 μU insulin per 100 g rat weight

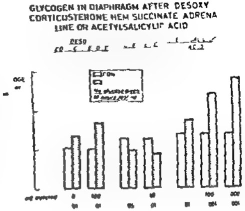

Fig 7 Glycogen content of rat diaphragm 2 hours after intraperitoneal injection of 1 ml saline without or with desoxycorticosterone hemisuccinate in doses of 10 or 100 μg adrenaline 1 or 10 μg or acetylsalicylic acid 10 100 or 1 000 μg per 100 g weight to intact overnight fasted rats

It was next investigated whether other substances especially hormones were able to increase diaphragm glycogen after their i p injection Growth hormone and glucagon administered i p both produce remarkable increases in diaphragm glycogen (Fig 5 and 6) although the doses injected were considerable Preparations of both bovine and human growth hormone are effective on a weight basis bovine growth hormone is the more active Whereas growth hormone in the doses administered has no effect on blood glucose values glucagon in doses of 100 and 1 000 μg per 100 g rat weight increases blood glucose to some extent

Desoxycorticosterone hemisuccinate and acetylsalicylic acid increase diaphragm glycogen after i p injection (Fig 7) adrenaline reduces it The last mentioned substances have no influence on blood glucose values in the doses applied

Discussion

The results reported illustrate the paramount importance of the route of administration of insulin This has been stressed by Madison and Unger (1958) in their work describing a direct action of insulin on the liver The change in response of different tissues as a result of a change of route of administration is closely related to the problem of binding of insulin either to tissues or to blood constituents Binding of insulin to a tissue was first clearly demonstrated by Stadie and his group (1949 a and b 1952) Dippling of isolated rat hemidiaphragm in insulin containing solution for 10 sec led to attachment of insulin to the tissue and several washes in buffer were unable to remove the insulin as demonstrated by increased glucose uptake by the hemidiaphragm during subsequent incubation This finding has been confirmed by other workers and extended to fat tissue in experiments with adipose tissue (Haugaard and March

1952 Humbel and Renold 1962) The question of insulin binding to one or more plasma fractions is still under debate but it is evident that insulin binding to plasma proteins and release from such binding may be of major importance as to when and where an action of insulin is observed Different modes of administration of insulin may decide to what extent and in what form the injected insulin is bound The speed of administration as well as the route of administration were important factors in the experiments of Madison, Unger and Renez (1960) and it was the combination of the intraportal route and slow infusion speed which enabled these workers to demonstrate a direct effect of insulin on glucose release from liver

The state of activity or rest of a particular organ and thereby its blood flow may also be of major importance when an effect of insulin is investigated In a resting animal the heart and the diaphragm have many more open capillaries at a given time than for example the muscles of the back or the limbs If insulin is bound to the tissues it seems likely that diaphragm and heart on weight basis will bind much more insulin than the resting muscles as the exposure of the tissue fibers to insulin is likely to be much more intensive in the active than in the resting muscles due to the greater blood flow in the former A demonstration *in vivo* in a resting animal of a direct insulin action on muscle would therefore *a priori* seem more feasible in muscles like heart and diaphragm than in skeletal muscles of the trunk and limbs This assumption was confirmed by the present experiments since significant changes in the amount of glycogen present in hind limb muscle was never observed either after intraperitoneal or after intravenous injection of insulin After i v injection of 10 000 μU insulin a moderate increase in glycogen was observed both in diaphragm and in heart pointing to a moderate insulin effect on both of these organs The picture changed completely when the same amount of insulin was administered by the i. p route A pronounced effect of insulin on diaphragm glycogen was now observed and in these experiments no increase in heart glycogen was seen Furthermore the change in blood glucose after i p injection of 10 000 μU insulin or less per 100 g rat weight was never of such a magnitude that it obtained statistical validity whereas the fall in blood glucose after i v injection of the same amount of insulin was always significant These observations form the basis for the following hypothesis After i p injection of small amounts of insulin the hormone is bound to the serosal surfaces lining the peritoneal cavity Insulin influences the metabolism of some of the tissues lining the peritoneal cavity but it has no effect on tissues out of contact with peritoneal coverings since insulin in these amounts does not reach the systemic circulation to an extent sufficient for a systemic effect. In the experiments presented here insulin increased glycogen of rat diaphragm but not that of rat epididymal fat tissue even though the latter also lines the peritoneal cavity and also exhibits extreme sensitivity to insulin In experiments to be reported (Rafaelsen, Lauris and Renold 1964) it was found by a tracer technique that incorporation of labeled glucose into glycogen

and fatty acid of epididymal fat tissue *in vivo* was indeed sensitive to small amounts of insulin after i p but not after i v administration With this information at hand it can be concluded that insulin after i p injection leads to an increased rate of glucose turn over in both diaphragm muscle and epididymal fat but that this results in a net glycogen increase only in diaphragm

Working with rat diaphragm *in vitro* several investigators have found that glucose uptake is a more sensitive index of insulin action than glycogen synthesis glucose uptake being increased by insulin concentrations as low as 10 to 100 μU/ml (Vallance Owen and Hurlock 1954 Rafaelsen 1957) whereas glycogen increase was only observed with insulin concentrations of 1 000 to 10 000 μU/ml (Stadie and Zapp 1947 Rafaelsen and Clausen 1961) Jessup and Wiberg (1961) were however able to obtain a glycogen increase in pieces of isolated diaphragm incubated *in vitro* in the presence of as little as 25 μU/ml insulin The insulin effect on glycogen content of diaphragm obtained *in vivo* in the present experiments is however greater than any reported from *in vitro* experiments It has not been investigated whether this greater insulin sensitivity was related to the better over all vitality of the tissue or to the diaphragm s rhythmic state of contraction and relaxation

The i p administration of growth hormone glucagon or desoxycorticosterone led also to an increase in diaphragm glycogen The doses given were much greater than those of insulin The greater effect of bovine than of human growth hormone might be interpreted as due to greater purity of the bovine preparation used It should be noted that a systemic influence cannot be excluded in the glucagon experiments since blood glucose levels were altered indicating an effect outside the peritoneal cavity in these experiments It should also be remembered that all glucagon preparations carry a minute amount of insulin A contamination of 0 4 % would give 100 μU insulin per 1 μg glucagon and might explain the increase in glycogen incorporation after i p injection as due to insulin contamination of the glucagon Adrenaline led not unexpectedly to a decrease in diaphragm glycogen Acetylsalicylic acid led to an increase It is natural to connect the last finding to the reports of fall in blood glucose in diabetic patients after administration of salicylates (Gilgore and Rupp 1961)

One of the reasons for studying other hormones than insulin was to see whether i p administration of these hormones in amounts likely to be found in 1 ml of human serum would influence glycogen content of rat diaphragm *in vivo* The results seem to indicate that amounts greater than those encountered in human serum are needed to obtain an effect This finding made it tempting to try to use i p studies for insulin assay of human serum The results obtained is reported in a following paper (Rafaelsen 1964)

More generally these studies with the i p administration of insulin suggest that the relative *in vivo* isolation of the tissues lining the peritoneal cavity as provided by this approach may prove useful for the experimental investigation of the mode of action of biologically effective agents

This paper was written while holding a travelling f llowship from Medical Research Cyanamid International Pearl River N Y USA
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The analytical work was performed by Mrs Bodil Kj r Kaltoft and Mrs Esther Mo gaard
Glucagon was generously supplied by Eli Lilly & Co Indianapolis Indiana growth hormone by Dr P J Randle Department of Biochemistry Cambridge University Cambridge England and by Dr C H Li Endocrine Research Laboratories Life Sciences Building University of California Berkeley 4 California USA.

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Acta physiol scand 1964 61 323—330

From Nordisk Insulin Laboratorium Gentofte and the 2nd Department of Internal Medicine Aarhus Municipal Hospital Aarhus University School of Medicine Aarhus Denmark

Insulin like Activity of Human Serum Determined by Glycogen Increase of Diaphragm after Intraperitoneal Injection into the Intact Rat

By

OLE J RAFAELSEN

Rec ved 6 December 1963

Abstract

RAFAELSEN O J *I sulin like activity of h man serum determined by glycogen in crease of diaphra m after intraperitoneal j ction into th intact rat* Acta phy ol scand 1964 *61* 323—330 — Intrap r toneal inj ction of 1 ml undil ited human serum in 100 g intact rats leads to increase of diaphragm glycogen On the basis of earlier investigat ons this can be assumed to represent i sulin activity pres nt in serum The glycogen increase caused by serum was graphi cally interpolated in a log/log system between increases cause l by 100 ar d by 10 000 μU insul n respectively Tw nty era from 11 normal fasting per sons had an av rage insul n like activity of 18(, U/ml rang 0 to 120 Nine sera from 6 fasting patients with prov n insulinoma had an av rage insulin l ke act vity of 583 μU/ml rang 14o to 1 520

The advantages of the method are a higl er specificity and gr ater simplicity than most of the *in vit o* methods used for m asuring serum insulin l k activity Its disadvantag is its lack of pr cisi on which i as great or greater than that of the *i vit o* methods

Serum or plasma insulin assay has in general been conducted along two lines 1 Intravenous injection of serum into small mammals sensitized to in sulin by hypophysectomy adrenalectomy and similar procedures or 2 in cubation of isolated tissue samples such as rat diaphragm or rat epididymal fat tissue in serum and measurement of metabolic indices known to be in fluenced by the presence of insulin *in vitro*

The present study was initiated when it was realized that minute amounts of insulin (100 to 10 000 μU per 100 g rat weight) increase glycog n content of rat diaphragm after intraperitoneal inj ction into intact rats with ut having detectable effects on blood glucose or glycogen content of muscular ti ue not lining the peritoneal cavity Raf elsen 196) This re ult effect r muscle

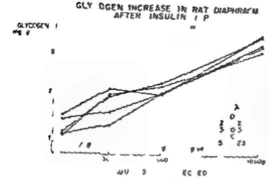

Fig 1 Glycogen increase of rat diaphragm 2 1/2 hours after intraperitoneal injection of 1 ml saline without or with 100 300 1 000 or 10 000 μU insulin per 100 g weight to intact overnight fasted rats Five consecutive expts Log/log system

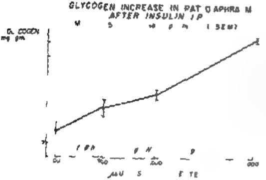

Fig 2 Glycogen increase of rat diaphragm 2 1/2 hours after intraperitoneal injection of 1 ml saline without or with 100 300 1 000 or 10 000 μU insulin per 100 g weight to intact overnight fasted rats Mean of 5 consecutive expts shown in Fig 1 (± S E M) Log/log system

calized yet *in vivo* obtained with amounts of hormone of the same order of magnitude as the amounts present in 1 ml human blood opened the possibility of obtaining information on insulin like activity of human serum by an assay combining some of the features of both the *in vivo* and *in vitro* procedures

Experimental

For details of procedure the reader is referred to a foregoing paper (Rafaelsen 1964) Only changes from earlier procedures and certain crucial features will be mentioned here

Procedure Only female albino rats (Wistar strain) weighing 80 to 120 g were used The animals were fasted for 18 hours with free access to water I p injection and sampling of tissues 150 min later (under carbon dioxide anesthesia) was performed as earlier described

In each experiment 16 to 24 rats were used divided into groups of 4 A control group was always injected with 1 ml 0 9 % saline Other groups were injected with saline containing glucagon free insulin (27 U/mg) in amounts from 100 to 10 000 μU per 100 g rat weight

Blood was drawn from fasted persons in the morning After 30 min at 4 C serum was separated by centrifugation The serum was always injected undiluted volume 1 ml Both serum which had been frozen from days to weeks at — 18 C and fresh non frozen serum was used There was no apparent difference between results obtained with frozen

Table 1 Glycogen content of hemidiaphragms isolated from rats 2.5 hours after intraperitoneal injection of saline without or with 100 or 10 000 μU of insulin or after injection of human serum from fasting normal patients (serum volume always 1 ml). Interpolations of serum values in a log/log system between mg increase in diaphragm glycogen caused by 100 and 10 000 μU of insulin are also tabulated. Patients no. 12 and 13 were omitted from calculation of average value as they did not fulfil criteria for normality (see text)

Pat no	Diaphragm glycogen (Mean ± SEM) Saline injected (mg of glycogen/g of wet diaphragm)	100 μU insulin injected (mg of glycogen/g of wet diaphragm)	10 000 μU insulin injected (mg of glycogen/g of wet diaphragm)	Serum injected (mg of glycogen/g of wet diaphragm)	Serum insulin like activity (μU/ml)
1	0.81±0.07 (6)	1.41±0.08 (6)	5.58±0.38 (6)	2.03±0.09 (6)	245
1	0.78±0.05 (5)	1.36±0.04 (8)	5.29±0.43 (8)	1.60±0.18 (8)	180
1	1.59±0.23 (6)	1.62±0.11 (8)	5.63±0.63 (8)	2.16±0.25 (8)	140
1	1.35±0.09 (6)	2.18±0.10 (5)	7.81±0.48 (6)	2.43±0.31 (6)	215
1	2.48±0.12 (8)	3.32±0.17 (8)	7.80±0.50 (6)	4.85±0.12 (8)	420
2	1.13±0.09 (6)	1.54±0.18 (6)	4.98±0.55 (6)	1.89±0.25 (6)	160
2	1.38±0.15 (6)	1.74±0.19 (6)	5.03±0.32 (6)	1.17±0.09 (6)	<100
2	0.94±0.04 (6)	1.22±0.10 (6)	4.60±0.[illegible]0 (6)	1.11±0.02 (6)	<100
3	0.66±0.05 (9)	0.83±0.08 (8)	5.11±0.42 (8)	0.80±0.05 (10)	100
4	0.92±0.09 (8)	1.56±0.06 (8)	4.85±0.37 (8)	2.08±0.35 (8)	230
5	0.60±0.03 (4)	0.95±0.03 (4)	7.97±0.27 (4)	1.03±0.09 (4)	120
5	1.26±0.17 (6)	1.82±0.25 (6)	5.60±0.61 (5)	2.53±0.43 (6)	255
6	1.53±0.08 (6)	2.37±0.32 (6)	8.73±0.78 (6)	2.87±0.21 (6)	280
7	2.43±0.26 (8)	2.58±0.28 (8)	6.17±0.47 (8)	2.92±0.23 (7)	130
7	1.57±0.14 (8)	1.71±0.09 (6)	6.69±0.46 (8)	2.95±0.22 (8)	255
8	1.13±0.12 (10)	1.27±0.15 (8)	5.18±0.30 (8)	1.91±0.15 (10)	170
9	2.02±0.12 (8)	2.41±0.17 (8)	9.23±0.53 (8)	3.43±0.15 (8)	205
9	2.72±0.32 (7)	4.31±0.23 (8)	11.55±0.74 (8)	5.00±0.26 (8)	160
10	[illegible].34±0.12 (8)	2.50±0.10 (8)	5.11±0.34 (8)	3.24±0.18 (8)	365
11	2.36±0.07 (8)	3.01±0.21 (8)	7.24±0.35 (8)	3.63±0.31 (8)	195
				Average	196
				Range	0–420
12	0.99±0.06 (10)	1.21±0.12 (8)	6.81±0.38 (8)	5.60±0.24 (10)	5 000
12	0.86±0.11 (8)	1.11±0.09 (8)	6.41±0.49 (8)	6.85±0.47 (8)	10 000
13	2.49±0.14 (8)	4.35±0.26 (8)	9.70±0.58 (8)	12.92±1.10 (8)	10 000

Number of hemidiaphragms given in parentheses

and with fresh serum. No attempt was made to correct for the presence of glucose in human serum by adding glucose to the insulin standards injected in saline.

Analytical methods. Glycogen content and blood glucose were determined as earlier described. Since blood glucose values between rats injected with serum, rats injected with insulin standards and rats injected with saline alone never differed significantly the results are not shown.

Calculation and statistical analysis. Each group of uniformly treated animals in an experiment consisted of 3 to 5 rats. From each animal both hemidiaphragms were sampled

Table II Glycogen content of hemidiaphragms isolated from rats 2.5 hours after intraperitoneal injection of saline without or with 100 or 10 000 μU of insulin or after injection of human serum from fasting patients with insulinoma (serum volume always 1 ml) Interpolations of serum values in a log/log system between mg increase in diaphragm glycogen caused by 100 and 10 000 μU of insulin are also tabulated The 4 experiments given below the calculated average are from 3 of the patients after extirpation of insulinoma

Pat no	Saline injected (mg of glycogen/g of wet diaphragm)		100 μU insulin injected (mg of glycogen/g of wet diaphragm)		10 000 μU insulin injected (mg of glycogen/g of wet diaphragm)		Serum injected (mg of glycogen/g of wet diaphragm)		Serum insulin like activity (μU/ml)
	Diaphragm glycogen (Mean ± SEM)								
14	1.32±0.11	(6)	1.28±0.08	(7)	6.83±0.33	(8)	3.48±0.29	(6)	600
14	1.70±0.09	(8)	4.01±0.42	(8)	9.0[illegible]±0.9[illegible]	(8)	4.44±0.3[illegible]	(8)	145
14	2.01±0.03	(8)	3.12±0.19	(8)	7.36±0.36	(6)	4.60±0.34	(8)	500
14	1.95±0.12	(6)	3.28±0.32	(8)	10.21±0.49	(8)	4.17±0.50	(8)	185
15	1.39±0.09	(6)	1.42±0.16	(8)	7.08±0.[illegible]8	(6)	2.68±0.23	(8)	205
16	2.37±0.12	(8)	3.20±0.18	(8)	7.16±0.49	(8)	4.52±0.13	(8)	430
17	2.12±0.13	(8)	3.28±0.21	(8)	7.03±0.44	(8)	5.48±0.11	(8)	1,570
18	2.14±0.19	(8)	2.9[illegible]±0.28	(8)	7.89±0.[illegible]1	(8)	5.24±0.44	(8)	1,220
19	2.44±0.09	(8)	2.69±0.17	(8)	7.76±0.47	(8)	3.05±0.20	(8)	2[illegible]0
							Average		583
							Range		145–1 570
14	2.3[illegible]±0.14	(8)	2.99±0.11	(8)	8.45±0.58	(6)	3.48±0.18	(8)	165
16	2.44±0.09	(8)	2.69±0.17	(8)	7.[illegible]6±0.6[illegible]	(6)	2.76±0.27	(8)	110
16	1.70±0.14	(8)	2.83±0.16	(8)	7.19±0.46	(8)	3.16±0.23	(6)	140
17	2.52±0.14	(8)	4.13±0.15	(8)	8.11±0.43	(8)	3.[illegible]9±0.20	(8)	<100

Number of hemidiaphragms given in parentheses

and mean values were thus based on 6 to 10 estimations The average glycogen increase in mg/g of wet diaphragm over the average value of the control group was plotted in a log/log system The average value of the serum injected group was in all experiments interpolated in this system between the glycogen increase caused by 100 and by 10 000 μU of insulin per 100 g rat weight respectively All values in the tables and figures are given ± S E M (standard error of mean) S E M never exceeded 15 The significance of difference between means has been established by calculating t P the probability of difference being due to chance was obtained from tables for t (Fisher and Yates 1943) λ, the index of precision was calculated in some of the experiments where four point standard curves were obtained

Results

The dose response relationship between insulin dose injected i p and diaphragm glycogen increase obtained is seen in Fig 1 and 2 The results are plotted in a log/log system The curves obtained in 5 consecutive experiments (Fig 1) demonstrate day to-day variation both in glycogen increase and in

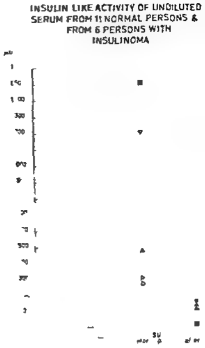

Fig 3 Insulin like activity of undiluted serum from fasting normal persons and from fasting patients with insulinoma before and after extirpation of insulin producing tumor

the relative linearity of the curves Accordingly the indices of precision yield rather high values indicating a considerable scatter of the results on which each curve is based The mean of the 5 expts (Fig 2) shows a definite dose response relationship in the range from 100 to 10 000 μU insulin injected per 100 g rat weight

Table I shows the values of insulin like activity of 20 serum samples from 11 fasting normal persons In the 3 expts where glycogen increase after injection of 1 ml serum was less than after injection of 100 μU insulin the activity obtained has been recorded as nil in calculating average and range The average of these 20 sera was 186 μU/ml human undiluted serum with a range from 0 to 420 μU/ml At the bottom of Table I 3 experiments are listed where very high values for insulin like activity were found Person no 12 was treated with high doses of acetylsalicylic acid (6 g per day) for backache of unknown etiology person no 13 was pregnant (first trimester) at the time of the investigation For these reasons these determinations were excluded from the normal group

Table II shows the insulin like activity of 9 serum samples from 6 fasting patients who all successfully had an insulinoma removed after completion of the investigations The average of these 9 sera was 583 μU/ml Table II and Fig 3 further show that in 3 of the 6 patients serum insulin like activity at determination was in the normal range The serum of one of the 3 patients was examined

Table II Glycogen content of hemidiaphragms isolated from rats 2.5 hours after intraperitoneal injection of saline without or with 100 or 10 000 µU of insulin or after injection of human serum from fasting patients with insulinoma (serum volume always 1 ml) Interpolations of serum values in a log/log system between mg increase in diaphragm glycogen caused by 100 and 10 000 µU of insulin are also tabulated The 4 experiments given below the calculated average are from 3 of the patients after extirpation of insulinoma

	Diaphragm glycogen (Mean ± SEM)								Serum insulin like activity
Pat no	Saline injected (mg of glycogen/g of wet diaphragm)		100 µU insulin injected (mg of glycogen/g of wet diaphragm)		10 000 µU insulin injected (mg of glycogen/g of wet diaphragm)		Serum injected (mg of glycogen/g of wet diaphragm)		(µU ml)
14	1.32 ± 0.11	(6)	1.28 ± 0.08	(7)	6.83 ± 0.33	(8)	3.48 ± 0.29	(6)	660
14	1.70 ± 0.09	(8)	4.01 ± 0.42	(8)	9.06 ± 0.95	(8)	4.44 ± 0.36	(8)	145
14	2.01 ± 0.08	(8)	3.12 ± 0.19	(8)	7.36 ± 0.36	(6)	4.60 ± 0.34	(8)	500
14	1.95 ± 0.12	(8)	3.28 ± 0.32	(8)	10.21 ± 0.49	(8)	4.17 ± 0.50	(8)	185
15	1.39 ± 0.09	(6)	1.42 ± 0.16	(8)	7.08 ± 0.58	(6)	2.68 ± 0.23	(8)	965
16	2.37 ± 0.12	(8)	3.20 ± 0.18	(8)	7.16 ± 0.43	(8)	4.52 ± 0.13	(8)	410
17	2.12 ± 0.13	(8)	3.28 ± 0.21	(8)	7.03 ± 0.44	(8)	5.48 ± 0.11	(8)	1,570
18	2.14 ± 0.19	(8)	2.92 ± 0.28	(8)	7.89 ± 0.51	(8)	5.24 ± 0.44	(8)	1,250
19	2.44 ± 0.09	(8)	2.69 ± 0.17	(8)	7.76 ± 0.47	(8)	3.05 ± 0.20	(8)	270
							Average		593
							Range		145–1,570
14	2.32 ± 0.14	(8)	2.99 ± 0.11	(8)	8.45 ± 0.58	(6)	3.48 ± 0.18	(8)	165
15	2.44 ± 0.09	(8)	2.69 ± 0.17	(8)	7.76 ± 0.62	(6)	2.76 ± 0.27	(8)	110
16	1.70 ± 0.14	(8)	2.83 ± 0.16	(8)	7.19 ± 0.46	(8)	3.16 ± 0.23	(6)	140
17	2.57 ± 0.14	(8)	4.13 ± 0.15	(8)	8.11 ± 0.43	(8)	3.79 ± 0.20	(8)	<100

Number of hemidiaphragms given in parentheses

and mean values were thus based on 6 to 10 estimations The average glycogen increase in mg/g of wet diaphragm over the average value of the control group was plotted in a log/log system The average value of the serum injected group was in all experiments interpolated in this system between the glycogen increase caused by 100 and by 10 000 µU of insulin per 100 g rat weight respectively All values in the tables and figures are given ± S E M (standard error of mean) S E M never exceeded 15 % The significance of difference between means has been established by calculating t P the probability of difference being due to chance, was obtained from tables for t (Fisher and Yates 1943 λ, the index of precision, was calculated in some of the experiments where four point standard curves were obtained

Results

The dose response relationship between insulin dose injected i p and diaphragm glycogen increase obtained is seen in Fig 1 and 2 The results are plotted in a log/log system. The curves obtained in 5 consecutive experiments (Fig 1) demonstrate day to-day variation both in glycogen increase and in

present method used one treatment for each group of 4 rats and gave consequently no information on intra individual variation apart from the duplicate determination Due to the limited degrees of freedom in this type of experiment a calculation along the lines of Renold *et al* was only performed in a few experiments It gave no further information than the simple graphical interpolation in a log/log system after calculation of mean and S E M and was therefore abandoned It should be mentioned that different systems of reference were tried and that a log/log system seemed to be the most suitable in the present rat diaphragm *in vivo* experiments as in experiments with isolated rat epididymal fat tissue *in vitro*

The index of precision was rather high with the present method indicating a considerable scatter of the individual results already when pure insulin solutions were injected It was therefore to be expected that the estimations of serum insulin like activity at best would show a similar scatter It is of interest under these circumstances that the results obtained are in agreement with results obtained by conversion of labeled glucose to carbon dioxide by isolated rat epididymal fat (Renold *et al* 1960) The values are somewhat higher than those obtained by other workers using the rat epididymal fat (Lyngsoe 1962) and by some workers using glucose uptake by isolated rat diaphragm (Vallance Owen and Hurlock 1954 Wright 1957) These latter values are again higher than values obtained by an isotope method employing antigen antibody reactions (Yalow and Berson 1960) With the available information on the limitations of all these methods the relative conformity of the results are more surprising to the author than the divergences Detailed discussions of these have been published and need not be repeated here It should be mentioned that findings of normal serum insulin like activity values in some cases of insulinoma have been reported by several investigators The excessive values in the two persons omitted from the calculations in the group of normal persons remain to be explained It was not possible to retest sera from these persons while this investigation was in progress

The present method has thus confirmed results of serum insulin like activity of human serum obtained by various *in vitro* methods Its advantages are 1 that it adds another and perhaps somewhat more specific parameter for insulin action to the ones commonly exploited in *in vitro* experiments and 2 its technical simplicity when compared with the *in vitro* methods in the latter especially the measurements of the small differences in glucose disappearance have been a stumbling block for investigators Its disadvantage is its lack of precision which is as great or greater than that of the *in vitro* methods It shares with the *in vitro* methods the overlap between normal sera and sera from patients with insulinomas and the occasional as yet unexplainable high value in individuals without evident disturbance of carbohydrate metabolism

The precision might be increased by using more animals for each assay This would not be an intolerable burden The animals can be kept in group after

the i p injection The chemical procedures are extremely simple and rapid including only weighing of the diaphragms boiling pH adjustment and glucose determinations

Determination of blood glucose during hypoglycaemia and after prolonged fasting remains however the most reliable test for insulinoma

The results obtained confirm the possibility of using i p injection and measurements of changes in the organs lining the peritoneal cavity in the study of drug and hormone action in a physiological setting

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Dr H C Hagedorn and Dr J E Poulsen Nordisk Insulinlaboratorium Gentofte and Dr K Lundbæk and Dr W Munck Aarhus University School of Medicine Aarhus, provided excellent laboratory facilities for the experiments The investigation was supported in part by grants from Landsforeningen for Sukkersyge Odense and from Købmand i Odense Johann og Hanne Weimann f Seedorffs Legat.

The analytical work was performed by Mrs Esther Mosgaard and Mrs Bodil Kjer Kaltoft.

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From the Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm 60, Sweden

The Effects of Extensor Muscle Spindles and Tendon Organs on Homonymous Motoneurones in Relation to γ-Bias and Curarization

By

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Abstract

Bianconi, R., R. Granit and D. J. Reis. *The effects of extensor muscle spindles and tendon organs on homonymous motoneurones in relation to γ-bias and curarization.* Acta physiol. scand. 1964. *61.* 331—347. — The ventral root L 7 is divided into a peripheral and central stump. The former is used for contracting the gastrocnemius soleus muscle by a brief tetanus supra threshold for the γ fibres. The latter records the monosynaptic test response by which the autogenetic excitability of the gastrocnemius-soleus motor nuclei is measured during and immediately after contraction. There are three major aims behind these experiments: (i) to discover whether at any time in or after contraction and *stretch the spindle secondaries can be given a functional task*, (ii) to provide a basis of comparison with similar experiments on flexors (subsequent paper) in which the spindle secondaries have autogenetic effects of opposite sign, (iii) to define autogenetic excitability during contractions in terms which are sufficiently precise to provide a basis for further work aiming at distinguishing between pre- and postsynaptic components of inhibition.

The old question of how sense organs measuring length and tension in the muscles contribute to reflex autogenetic control of their own muscles was clarified for extensors in general outlines fourteen years ago (Granit and Suursoet 1949, Granit 1950, Hagbarth and Naess 1950, McCouch, Deering and Stewart 1950, Granit and Ström 1951, Hunt 1952). The reason for returning to it now is the improved knowledge of the anatomy of the muscle spindles (Cooper 1959, 1960, 1961, Swett and Eldred 1960, Barker and Gidumal 1961, Barker and Cope 1962, Boyd 1962) which poses fresh problems. This paper will deal with limb extensors, a second paper (Bianconi, Granit and Reis 1964)

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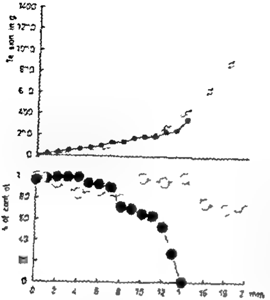


Fig 1 Different patterns of changes in excitability of gastrocnemius-soleus motoneurones during static stretch of de efferented gastrocnemius-soleus Extension in mm Two cats Nembutal anesthesia *Upper graph* Muscle tension at different extensions in the two animals *Lower graph* Monosynaptic excitability at different extensions Lines in full and broken lines in each graph corresponds to the same animal

whose peripheral portion had been chosen for stimulation of the muscle Paraffin oil was used to cover exposed tissues

The conditioning tetanus for the peripheral stump of the cut ventral root lasted 19 msec and consisted of a train of shocks of 0 8 msec duration delivered at a frequency of 500 sec Tetanic stimulation was used in order to produce a sufficient degree of intrafusal contraction when wanted Occasionally motor twitches were studied

The test stimulus to the gastrocnemius nerves consisted of a single shock of 0 9 msec duration set at an intensity which produced a monosynaptic test response of about 50 % of maximum

Muscular contraction was recorded by a strain-gauge myograph with a maximum sensitivity of 0 75 g mm deflection Muscle length and tension are referred in the paper to that found at zero length which is defined as that length of muscle at which the myograph at high sensitivity senses an increment of tension when the slack is taken off the muscle by pull on the tendon This zero length was frequently checked during the course of the experiment

In order selectively to block extrafusal contraction without affecting intrafusal contraction two techniques were used The first was an attempt to block the α motor fibres by anodal blockade in a manner similar to that described by Kuffler and Vaughan Williams (1953 by adjusting interelectrode distance shock strength and duration for the electrodes on the cut ventral root It actually proved possible on occasion to demonstrate that intrafusal contraction as deduced from increased discharge of single spindle afferents was proceeding with little or even no contraction of extrafusal fibres This dissociation between extra and intrafusal contraction was seen for both primary and secondary spindle afferents and from ankle flexor and extensor muscles This result proved to be highly variable however and success or failure in selective blockade depended on a number of critical factors which excluded regular use of anodal blockin Because of this inconstancy this approach was abandoned in favour of selective blockade by Flaxedil It is mentioned here because as far as we know it is first the time the method has been applied to the mammalian preparation with some success

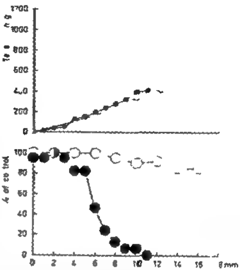


Fig 2 Spinal cat (Th 12) Changes in the excitability of gastrocnemius soleus motoneurones during static stretch of de efferented gastrocnemius-soleus Extension in mm Single and double monosynaptic test shocks *Upper graph* Muscle tension at different extensions in two trials with single (line in full) and double (broken line) test shocks *Lower graph* Monosynaptic excitability at different lengths with single (line in full) and double (broken line) test shocks

Blockade of extrafusal contraction was successfully accomplished by the intravenous administration of Flaxedil which in low doses may eliminate extrafusal contraction but leave intrafusal contraction unimpaired (Granit Homma and Matthews 1959) Doses of Flaxedil of 1 5—2 0 mg/kg given in a single intravenous injection predictably resulted in almost complete attenuation or total disappearance of all extrafusal contraction within 3 min after the injection This state persisted for 15—30 min while the animal was maintained on artificial respiration With this dose of Flaxedil the monosynaptic control did not change and the discharge of single spindles elicited by supramaximal stimulation of ventral roots was preserved

Results

Effects of static stretch on monosynaptic reflex excitability

It has been shown previously (Granit 1950 Granit and Strom 1951 Henneman 1951 Hunt 1952) that static stretch of the ankle extensors usually but not invariably will result in an inhibition of the monosynaptic reflex elicited from the homonymous and heteronymous muscle nerves These experiments have been repeated in order to evaluate the effect of steady stretch of ankle extensors at different extensions and hence at different tensions on the monosynaptic test response elicited homonymously All observations were made 20—30 sec after the muscles had been stretched to a given length and the monosynaptic test response had stabilized

In general plots of the monosynaptic reflex excitability against both extension and passive tension led to curves which fell into two broad groups illustrated in Fig 1 by two representative experiments The two curves are derived from two different animals both anaesthetized with Nembutal and whose passive tension curves upper graph) are almost identical In the first type (Fig 1

solid line) the monosynaptic test response either started at or reached its maximal response within the first 3—4 mm of stretch during which the tension in the muscle rarely exceeded 50 g With further stretch the monosynaptic test response became reduced with a tendency for the diminution of the response to increase progressively with each further mm of stretch until it disappeared completely The onset of the downward break in the curve was more consistently observed at tensions of 50—100 g than at any constant length

In the second type of response (Fig 1 broken line) monosynaptic excitability was not reduced by more than some 30 % by stretching the muscle, even beyond the physiological range In some instances no reduction of the monosynaptic test response was seen at all

It was found possible to convert the first type of curve (exhibiting sensitivity to inhibition) to that of the second type by doubling the monosynaptic test shock An example of this is illustrated in Fig 2 Initially the monosynaptic excitability to a single test shock (Fig 2 solid line) was progressively reduced by increasing stretch after exceeding a threshold *at about 50 g of tension and* hence progressed to complete inhibition at 11 mm of stretch when the tension was 400 g By then adjusting the intensity and duration of the test shock so that the test response was doubled and only the (summated) second shock resulted in a monosynaptic test response (Fig 2 broken line), the monosynaptic response *was only inhibited by 15 % even at extreme lengths and high tension*

Comment These experiments confirm earlier work (Granit and Strom 1951 Henneman 1951 Park Teasdall and Magladery 1951 Hunt 1952 Buller and Dornhorst 1955 Libet Feinstein and Wright 1959) and demonstrate inhibition in stretch except when double shocks are used whose synaptic potentials sum levels beyond those that can be prevented by inhibitory repolarization It is likely that both tendon organs and spindle secondaries contribute their share to such inhibitions as have been seen here during static stretch with tensions of over 50 g which is the beginning of the range for stimulation of tendon organs Such tensions represent extensions well beyond the threshold for secondary spindle organs and strongly suggest that in the circumstances inhibition from secondaries requires the support of the tendon organs to exceed the concealed state The different types of curve obtained are best explained by *the settings of spinal internuncial control* as determined by supraspinal and other sources Such factors have been studied by Job (1953), R M Eccles and Lundberg (1959) Holmqvist and Lundberg (1959) Holmqvist, Lundberg, and Oscarsson (1960) Hufschmidt (1960 1961)

Effects of contraction on monosynaptic reflex excitability

It is convenient for the purpose of relating the changes in monosynaptic reflex excitability to changes in the afferent input arising from the contracting muscle to divide the events from stimulus to contraction into four phases (see Fig 8) as follows *I* a pre contraction phase consisting of the period from the

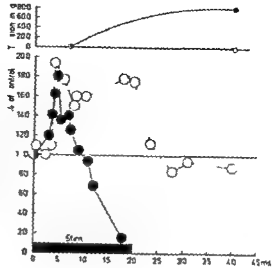


Fig. 3. Cat. Nembutal anesthesia before and after Flaxedil. Excitability of gastrocnemius motoneurones and muscular response conditioned by tetanization of distal end of cut ventral root L7. Muscle extended 2 mm. Stimulus 10 × threshold for extrafusal contraction. Timing in msec begins with onset of tetanization. *Upper graph*: Muscle tension before (line in full) and after (broken line) Flaxedil (2.0 mg/kg i.v.). *Lower graph*: Monosynaptic excitability before (line in full) and after (broken line) Flaxedil.

onset of ventral root stimulation to the earliest observable contraction of the muscle as recorded by the myogram set at moderate or high gain. *II*, a phase of tension rise which begins at the moment of earliest observable contraction, as above, up to and through the plateau of maximal tension occurring during a tetanic stimulation. *III*, a phase of declining tension beginning at the first fall-off of muscle tension from its peak value during relaxation and terminating when muscle tension has returned to its pre-contraction level. *IV*, a late or post-contractile phase beginning at the termination of any recorded tension. The changes that occur in monosynaptically tested excitability during these events will now be described for each phase separately.

I. Pre-contraction phase. The presence of facilitation of the monosynaptic test response beginning 3—4 msec following a supramaximal conditioning stimulation of the cut ventral root and prior to the onset of contraction, was demonstrated by Granit (1950) and confirmed by Hagbarth and Naess (1950). In this study a similar early facilitation of the monosynaptic test response was commonly observed. This exhibited a latency from the onset of a tetanus as brief as 3 msec and persisted during the stimulus train until the beginning of the muscle contraction. Then it rapidly declined or disappeared. Markedly reducing or totally abolishing extrafusal muscle contraction by the administration of intravenous Flaxedil did not alter the magnitude of it. After significant reduction or elimination of contraction by this technique, the early facilitation persisted until several milliseconds after termination of stimulation of the ventral root as illustrated in Fig. 3. It should be noted that although the fall of the reflex excitability precedes the onset of observed contraction by 2 msec, the possibility of some contraction preceding its registration by the myograph is real. In this

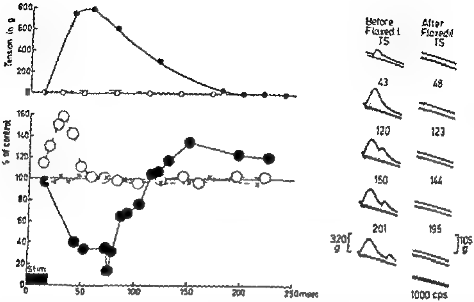


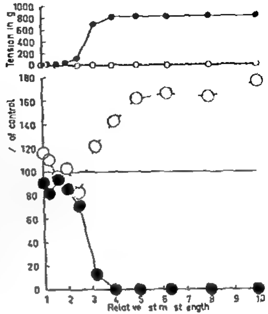
Fig 4 Spinal cat (C 1) before and after Flaxedil Excitability of gastrocnemius motoneurones and muscular response as conditioned by tetanization of distal end of cut ventral root L7 Resting length of muscle 10 mm Stimulus at 3 2 × threshold for extrafusal contraction Timing in msec begins with onset of tetanization *Upper graph* Muscle tension before (line in full) and after (broken line) Flaxedil *Lower graph* Monosynaptic excitability before (line in full) after Flaxedil (broken line) and after section of muscle nerve (crosses) *Inset* Samples of original records of myogram (upper line) and monosynaptic test response (lower line) with test shock (TS) alone with conditioning shocks at times marked above them Tests after Flaxedil begin at 4 min after injection of 2 0 mg/kg i Note that Phase I is not included in the figure

instance the latter was set at a moderate sensitivity As a rule the fall of reflex excitability ran parallel with the rise of muscle tension

It appears therefore that the decline of the Phase I facilitation is due to the alteration in input to the spinal cord from muscle receptors and is dependent on contraction The mechanism of Phase I facilitation of the monosynaptic test response its relationship to the early discharge and its implications with regard to α motor innervation of the muscle spindles have been discussed in detail by others (Granit Pompeiano and Waltman 1959 Hunt and Perl 1960 Bessou Emonet Denand and Laporte 1963) The present study has not been designed critically to examine this particular problem However the persistence of Phase I facilitation after paralytic doses of Flaxedil makes its dependence wholly on tensile or ephaptic excitation of afferents from the massed muscle action potential unlikely

II Phase of increasing and maximal muscle tension This as we have seen is accompanied by a considerable fall of excitability in Fig 4 beginning in spite of maintained supramaximal stimulation of the ventral root These observations confirm those previously made in this laboratory (Granit and Suursoet 1949 Granit 1950 Granit and Strom 1951 Hagbarth and Naess

Fig 5 Same cat as Fig 4 before and after Flaxedil Changes in excitability of gastrocnemius motoneurones at different intensities of conditioning tetanus to distal end of cut ventral root L7 Intensities in multiples of threshold intensity for extrafusal contraction Interval between onset of conditioning tetanus and test shock 42 msec. Resting length 10 mm *Upper graph* Maximal recorded tension of gastrocnemius during contraction before (line in full) and after (broken line) Flaxedil (2 0 mg/kg i v) *Lower graph* Monosynaptic excitability before (line in full) and after (broken line) Flaxedil



1950) Curarizing by Flaxedil so as to remove tension while retaining for a while intrafusal sensitivity to motor impulses led to the striking change seen in Fig 4 Phase II inhibition reverted to a facilitation which gradually approached zero The late facilitation belonging to a later phase (see below) disappeared The duration of the Phase II facilitation under Flaxedil varied from case to case maximally it lasted up to 220 msec into Phase III and increased with increased resting length It should be noted that no observations in Fig 4 refer to Phase I The experiments generally ended with severance of the motor nerve to prove that the effects noted were muscular

Stimulus strength and extension determine the magnitude of the motor effect on intrafusal fibres and so for Fig 5 we have selected the moment of near maximum facilitation (as determined after Flaxedil) for a study of the effect of the intensity of stimulation at an extension of 5 mm empirically found to be sufficient for good spindle activation The interval between onset of ventral root tetanus and test shock was 42 msec at that moment The abscissae in Fig 5 represent stimulus strength in multiples of threshold for extrafusal contraction

It is seen in the figure that before Flaxedil inhibition by our test essentially ran parallel with contractile tension but that after Flaxedil leading to removal of tension the remaining facilitation required stimuli of a strength near the extrafusal maximum in order to appear at all and that it continued to increase when the stimuli became stronger (5 x threshold) This of course is as would

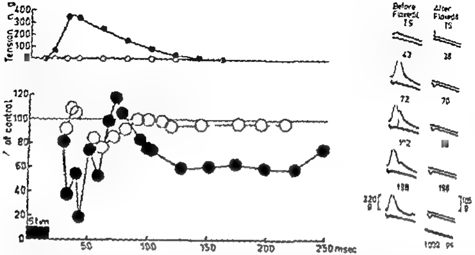


Fig 6 Spinal cat (C 1) before and after Flaxedil Excitability of gastrocnemius motoneurones and muscular response as conditioned by tetanization of distal end of cut ventral root L7 Zero resting length of muscle Stimulus 10 × threshold for extrafusal contraction Timing in msec begins with onset of tetanization *Upper graph* Muscle tension before (line in full) and after (broken line) Flaxedil *Lower graph* Monosynaptic excitability before (line in full) and after Flaxedil (broken line) *Inset* Samples of original records of myogram (upper line) and monosynaptic test response (lower line) with test shock (TS) alone and with conditioning shocks at times marked above them Tests after Flaxedil begin at 4 min after injection of 2 0 mg kg i v

be expected from present knowledge of the role of the γ fibres for spindle primaries and thus indirectly for their reflex effects (see e g Granit 1955)

Such experiment as a rule ended with controls in which either procaine block or nerve section was used to demonstrate that the ventral root stimuli actually had activated the intrafusal muscle fibres of spindles in the ankle extensors

It is of interest that although intrafusal contraction initiated by stimulation of γ motor fibres is well known to result in increased discharge from excitatory primary and inhibitory secondary spindle endings facilitation uniformly dominated after Flaxedil paralysis This indicates that the net result of augmenting spindle discharge by this method of γ motor fibre stimulation will always be to produce more primary than secondary effects no matter what the initial muscle length or tension and this holds good even in animals of the type showing failure of inhibition to extreme stretch The regularly occurring inhibition during this phase of contraction also provides further documentation in support of the view that autogenetic inhibition during contraction is the dominant effect of tendon organs rather than merely the result of withdrawal of autogenetic facilitation by spindle unloading Supramaximal tetanic stimulation should maintain some spindle activity within the pause (Hunt and Kuffler 1951 *a*)

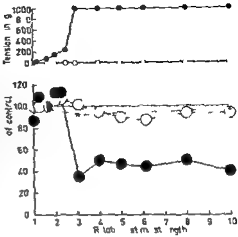


Fig 7 Same cat as Fig 6 before and after Flaxedil Changes in excitability of gastrocnemius motoneurones at different intensities of conditioning tetanus to distal end of cut ventral root L7 Intensities in multiples of threshold intensity for extrafusal contraction Interval between onset of conditioning tetanus and test shock 100 msec Resting length zero *Upper graph* Maximal recorded tension of gastrocnemius during contraction before (line in full) and after (broken line) Flaxedil (7 0 mg/kg i v) *Lower graph* Monosynaptic excitability before (line in full) and after (broken line) Flaxedil

III Phase of excitability during declining tension During the period of decline from the peak tension excitability usually begins to increase Though the change in the curve temporally corresponds to the onset of decline of peak tension its rate of return to control levels is faster than that of muscle tension (Fig 6) Frequently this effect has the character of a transient facilitation which occurs when the muscle tension has fallen to 1/2 or 1/3 of its peak tension (see Fig 4 and 6) This transient facilitation has been previously noted (Granit 1950) Its relationship to the time course of relaxation of the muscle is identical with that of the small contraction seen during the period of declining tension of the tendon jerk the so-called myotatic hump (Creed Denny Brown Eccles Liddell and Sherrington 1932) or myotatic appendage (Ballif Fulton and Liddell 1925) and it would thus appear to be the neuronal basis of that mechanical event Despite loss of tension the late facilitation usually ends with reversal to inhibition (see Fig 6) Less frequently facilitation persists (Fig 4) Following the administration of Flaxedil the transient Phase III facilitation disappears completely while the Phase II facilitation which occurs during and immediately following the tetanic stimulation still is seen indicating that the absence of the response after small doses of Flaxedil is due to something caused by variations of muscle tension The transient Phase III facilitation may be seen with the muscle at any initial length although it generally tends to be accentuated by greater lengths

In some instances with prolonged inhibition the Phase III facilitation may be concealed and only appear as a transient reduction in inhibition The onset of this transient rise in excitability also coincides with the moment at which the myotatic hump occurs

IV Post contraction phase Changes in the amplitude of the test response are usually seen to persist following the return of the muscle to its control length and

tension. Most commonly during this period there is marked inhibition which may continue up to several hundred milliseconds after muscle tension has disappeared and hence, unlike the inhibition found during Phase II of contraction, is independent of tension. Nevertheless, following administration of Flaxedil this persistent late inhibition entirely disappears. This is illustrated in Fig. 6.

The relationship of the late persistent inhibition of Phase IV to the stimulus intensity is illustrated in Fig. 7, plotted as Fig. 5. The curves have been obtained from the same animal as illustrated in Fig. 6 and the interval between the onset of the conditioning tetanus and the test shock is chosen to be 100 msec which is well within the late persistent inhibition. It is seen that both the reflex inhibition (Fig. 7, lower graph, solid line) and the muscular contraction (upper graph, solid line) reach their plateaus at similar stimulus intensities — 3.2 × threshold — although the threshold for the onset of the monosynaptic reflex inhibition is somewhat higher than that for contraction. Following the elimination of extrafusal contraction by Flaxedil (Fig. 7, upper graph, broken line) both contraction and monosynaptic inhibition (Fig. 7, lower graph, broken line) disappear, an effect which also follows section of the muscle nerve.

The long lasting Phase IV inhibition may also follow a twitch contraction of the ankle extensors elicited by a single shock to the ventral root at the threshold for maximal extrafusal contraction. It is seen with muscles at any initial length or tension, although occasionally its magnitude may diminish at greater initial lengths.

γ On occasion a late persistent facilitation of monosynaptic reflex excitability has been found (Fig. 4) which then appears as a continuation of the facilitation that develops in Phase III with the fall off from peak tension in the relaxing muscle. This, too, usually disappears following Flaxedil although in one instance when the muscle was stretched to 10 mm it persisted after Flaxedil blending into the facilitation that occurred during and immediately following the tetanic conditioning stimulus.

Reversal of a Phase IV facilitation to inhibition was seen in one animal anaesthetized with Nembutal following spinalization at T12 later in the experiment. In another experiment Phase IV facilitation following a conditioning tetanus at 3.2 × threshold for extrafusal contraction reverted to inhibition when the intensity of the conditioning stimulus was raised to 10 × threshold without any observed change in the magnitude or duration of the muscle contraction. This reversal with increased conditioning stimulus intensities was an exception for in all other instances no significant alteration in the late response was observed for conditioning stimuli greater than those required for maximal extrafusal contraction.

It would appear therefore that Phase IV inhibition in some way depends on contraction. It is independent of tension and evidently its genesis is not fundamentally caused by prior excitation of γ motor fibres because it also occurs after a motor twitch.

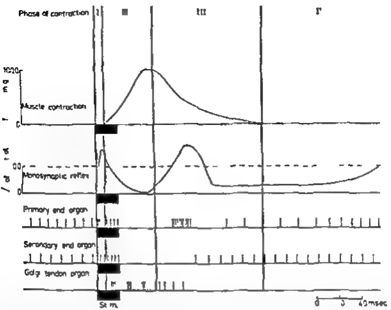


Fig 8 Schematic representation of temporal relationship between spindle and tendon organ response, excitability of ankle extensor motoneurones and contraction of ankle extensors initiated by tetanic stimulation of distal half of cut ventral root. The muscle is assumed extended to about 5 mm and peak contraction is about 1 000 g. The stimulus intensity is over 5 × threshold for extrafusal contraction. The phases of contraction (I—IV) are as described in text. Monosynaptic reflex is in relative magnitude of control. Receptor activity represented as discharge of single afferent units.

Discussion

Fig 8 summarizes our results and relates the four phases described to probable events in the end organs which measure muscle length and muscle tension during tetanization of the root (generally L 7) at strengths exceeding what is required for the γ fibres. The behaviour of spindles during such circumstances is reasonably well known and so the literature before 1955 need not be specifically quoted (summarized by Granit 1955). Autogenetic excitability of the motoneurones is plotted below the curve for contraction.

Phase I The early phase of facilitation which represents a kind of positive feedback mechanism helping the muscle to start was described by Granit and Suursoet (1949) and by Granit (1950). When ventral roots are stimulated there occurs a well known sensory backresponse (e g Lloyd 1941, 1942; Leksell 1945) which Hunt and Kuffler (1951 *b*) called the early discharge having analyzed it in terms of single spindle and tendon organ afferents. They ascribed it to mechanical events preceding recordable contraction. Granit et al (1959) proved that the earliest part of it is due to ephaptic stimulation as

Lloyd and Leksell had surmised This effect can hardly be avoided in extensors with shocks to the ventral roots and so, in the present experiments it must initiate Phase I facilitation Succeeding ephaptic stimulation in extensors and likewise in flexors were ephaptic effects are negligible, spindles fire before γ impulses have had time to influence them (Granit et al 1959) and do so also in response to reflex stimulation (Rutledge and Haase 1961) Granit et al (1959) failed to find satisfying correlations with tension and extension and therefore suggested that there would be α innervation of some spindles as since shown to exist by Bessou Emonet Denand and Laporte (1963) in the digital muscles where sufficient isolation of individual components can be obtained The functional significance of fast spindle activation is independent of whether the α fibres concerned go to spindles alone to extra and intrafusal muscle or only to nearby extrafusal fibres to which spindles are inserted so as to receive a brief twitch while the full force of the extrafusal contraction later is neutralized' (Granit et al 1959 p 416)

Phase II As pointed out above early spindle effects are neutralized during rising tension, partly by the pause of the spindles but definitely also by inhibition sensitive to tension and hence arising in tendon organs Thus at this stage brakes are applied (negative feedback)

Phase III The transient facilitation on the falling phase of contraction which corresponds to the myotatic appendage or hump of the tendon jerk (see above) has a natural explanation in the fact that a considerable number of spindle primaries deliver a high frequency burst at the moment of drop in tension (Granit and Van Der Meulen 1962) Therefore it is — and should be — absent when contraction is removed by Flaxedil

Phase IV Of great interest is the late inhibition which demonstrates that the balance between primaries and secondaries is shifted in favour of the latter because tension now is negligible After an isometric contraction those primaries which possess a transient burst in Phase III are silent for a while afterwards before they again pick up their discharge (Matthews 1933 Harvey and Matthews 1961 a Bessou and Laporte 1962) A large number of the others start firing gradually This is probably an expression of their great phasic sensitivity to small variations of length (Granit and Henatsch 1956) non existent in the large majority of secondaries (Bianconi and Van Der Meulen 1963) After an isometric contraction the latter fire at the rate determined by the degree of extension (Bessou and Laporte 1962 see especially their Fig 9) In this situation (Phase IV) the inflow from the secondaries dominates over that of the primaries as illustrated in Fig 8 and for this effect it is of little significance whether contraction is caused by a shock or by a tetanus

This being so, Flaxedil by removing the contraction and thus the cause of the post contractile depression of the discharge from the primaries must also remove the inhibition recorded in Phase IV, as demonstrated above (Figs 6 and 7)

The idea launched by Boyd (1962) that the nuclear chain fibres dominated by secondaries are activated by slower γ fibres (γ_2) than the nuclear bag fibres (γ_1) made us suspect that there would be more of the late inhibition the stronger the stimulation of the ventral roots This occurred only once (see above) and so we cannot say that our experiments have offered any evidence in favour of Boyd s notion Indeed none of the other inhibitions we have seen have ever been increased by increasing stimulus strength to the ventral root so as to bring in the smallest γ fibres Spinalization however which balances the spinal cord towards the flexor reflex and increases its sensitivity to extensor inhibitions (Job 1953 R M Eccles and Lundberg 1959 Holmqvist and Lundberg 1959 Hufschmidt 1960 1961) was found to emphasize concealed or weak inhibitions

Perusal of the descriptive sections will show that in every instance after Flaxedil when γ fibres were brought in by stimulation in excess of that needed for maximal contractions the autogenetic reflex excitability behaved as if primaries alone had been activated If this be due to the unphysiological mode of stimulation the more important it seems that natural dominance of the secondaries in Phase IV is a consequence of extrafusal contraction If this explanation of our finding is correct a consequence of autogenetic facilitation in flexor secondaries is that Phase IV must be excitatory in them This is actually the case as our second paper will demonstrate (Bianconi Granit and Reis 1964) and the opposite effects in extensors and flexors in Phase IV would seem to exclude after effects of engagement as such (see our second paper)

Thus for the first time a functional role of these elusive organs the spindle secondaries has been found with natural stimuli because extrafusal contractions can hardly be regarded as unnatural howsoever caused They must belong to the arsenal of behavioural patterns

Summary

1 In cats under Nembutal the nuclei of the ankle extensors have been conditioned by a contraction elicited from the cut peripheral stump of the appropriate ventral root by brief tetani above γ threshold The excitability of the nuclei has been studied by monosynaptic testing along the course of the contraction

2 Flaxedil has been used to remove contraction while leaving the effect of the γ efferents on the intrafusal muscles practically intact

3 The results are summarized and related to the contraction and the discharge of spindle primaries secondaries and tendon organs in Fig 11

4 The pause of reduced excitability of the extensor centre after contraction is ascribed to the spindle secondaries

5 The results should be compared with those obtained in similar experiments on the flexor centre in the subsequent paper

This work has been supported by the Swedish Medical Research Council and the Rockefeller Foundation

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Table I

	Spindle primaries	Spindle secondaries	Tendon organs
Extensors	Excitation	Inhibition	Inhibition
Flexors	Excitation	Excitation	Inhibition

cessation of spindle activity (the pause) and the post contraction inhibition by the relative dominance of spindle secondaries at this moment as demonstrated by Harvey and Matthews (1961) and Bessou and Laporte (1962) the latter stimulating γ fibres and recording from secondaries and primaries in the same spindle In referring below to our previous paper we shall call it Paper I

Now it is well known that the spindle secondaries which possess afferents in the Group II category (Merton 1953 Hunt 1954) are excitatory on the flexor motoneurones (Laporte and Lloyd 1952 Hunt 1954 Laporte and Bessou 1959) This means that while these receptors in extensors produce autogenetic inhibition they should in the flexors produce autogenetic excitation Thus in the latter one important source of inhibitory impulses from muscular afferents is replaced by a facilitatory source Golgi tendons organs on the evidence of Laporte and Lloyd (1952) would be inhibitory in flexors as they are in extensors at least to the extent that they belong to Group Ib More complex patterns of Ib action were described by Eccles Eccles and Lundberg (1957) but these do not seem to be autogenetic Spindle primaries are excitatory in both flexors and extensors This comparison between autogenetic effects of extensors and flexors is summarized in Table I o

From this table emerges that in extensors the autogenetic spindle effects are antagonistic in flexors synergistic Thus whenever both spindle organs are excited together one should expect excitation to dominate in flexors while in extensors this could be the case only if the autogenetic effects of the primaries for some reason were more potent than those of the secondaries Initially in contraction they proved to be so (Paper I) Later on in the post contraction phase (Phase IV of Paper I) a long lasting autogenetic inhibition was found This effect was ascribed to the secondaries The explanation presupposes that the equivalent phase in the flexors should be characterized by autogenetic excitation (cf Table I) One of the chief reasons for extending our work to flexors was to test this proposition

Methods

The technique and general procedure is identical with the one described in our previous paper on extensors except that in the present case denervation of the limb left the two flexors tib ant and extensor longus digitorum, with intact nerves The two muscles were separated and their tendons then joined together to the isometric myograph care being taken to adjust their lengths to approximately equal values The ventral

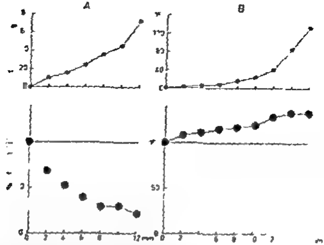

Fig. 1. Autogenetic effect of stretch on the monosynaptic reflex of ankle flexors in the intact (A) and spinal (B) animal. In both diagrams: Abscissae: extension in mm. Ordinate: lower curve amplitude of monosynaptic reflex in per cent of amplitude at initial zero length; upper curve muscle tension in g.

roots were severed up to and including L6, that of L7 cut in the middle so as to leave a peripheral stump for stimulating the muscle while the central stump was used for recording the monosynaptic response elicited from electrodes on the peroneal nerve at the level of the knee.

As in the previous experiments the animals were anesthetized with pentobarbitone; otherwise they were intact. Spinalization at the level of C1 was carried out in one case.

In all work of this character there is the complication of homosynaptic versus heterosynaptic testing, discussed at some length by Granit and Job (1952), but since our aim is to compare extensors with flexors under similar circumstances we have avoided this issue by using homonymous (homosynaptic) testing in both cases. Both types of experiment will then be contaminated by the same error caused by the afferent barrage in the largest fibres. Of particular interest is again the outcome of the test in the post-contraction phase because at that stage the half maximal monosynaptic test response should be a reliable measure of autogenetic excitability in the pools of both extensor and flexor motoneurones and in the two cases the autogenetic effects, as stated, would have to be in the opposite direction. Since the spindles pause during the rising phase of the contraction a comparison of flexors and extensors by homonymous testing would be reliable in this situation too.

Fig. 8 of Paper I subdivides the events from stimulus to end of contraction into four phases which will be used as standards of reference also in the present work.

Results

In the spinal animal with its flexor pool released Job 1953, R. M. Eccles and Lundberg 1959, Holmqvist and Lundberg 1959, Henneman 1951 found that stretch causes facilitation of the monosynaptic test response. We have confirmed this on the one spinal animal used whose curves for passive tension and motoneurone excitability are shown in Fig. 1 B. In the intact animals the effect of stretch turned out to be inhibition, just as in the extensors, and this

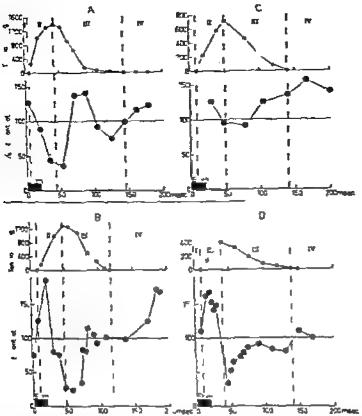

Fig 2 Time course of autogenetic effects of conditioning L7 stimulation 5 to 10 times a strength on monosynaptic reflex of ankle flexors in 4 different experiments. In each diagram Abscissae, time in msec from beginning of conditioning stimulation. Heavy line duration of conditioning stimulation at .00 sec and a supramaximal intensity Ordinate lower curve amplitude of monosynaptic reflex in per cent of its unconditioned amplitude upper curve muscle contraction in g I = pre-contraction phase II = contraction phase III = relaxation phase IV = post contraction phase Description in the text.

effect as a rule started at extensions as small as to be around 2 mm Occasionally excitability for the first 2 to 4 mm of extension rose a little but in the end inhibition prevailed The typical curve is shown in Fig 1 A lower graph)

While in the extensors the maximum inhibition brought the test response to zero at extensions around 12 to 14 mm in flexors the maximal inhibitions ranged from 50 to 80 % They were never complete

The difference between extensors and flexors is thus so to speak in the right direction in view of the known properties of their sensory afferents acting to determine autogenetic excitability (Table I) There are more inhibitory afferents in the extensors and the spindle secondaries whose activation requires more stretch than is necessary for the spindle primaries should in the flexors augment

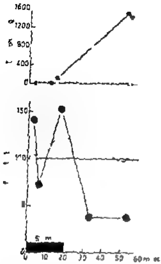

Fig 3 Example of facilitation in Phase I preceding the facilitation at onset of contraction and effect of Flaxedil upon it Abscissae time from commencement of conditioning stimulation Duration of this heavy line Ordinate lower curve amplitude of monosynaptic reflex in per cent of its unconditioned amplitude upper curve muscle contraction in g ● before and ○ after Flaxedil

autogenetic excitability and never diminish it The preparation needs but be balanced towards flexor dominance by spinal section to show the expected facilitation to stretch instead of the complete inhibition so characteristic for the homosynaptically tested extensors

Contraction

Survey A general survey is given in the four experiments plotted in Fig 2 all of which compare changes of tension (above) with changes of excitability (below) The stimulus to the ventral root was always supramaximal for α motor fibres as measured by the size of the contraction because, as in the experiments with the extensors activation of γ fibres was aimed at Initial tension was moderate just above the presumed threshold for the spindle secondaries The separation of the curves into the four phases marked I—IV, is taken from Fig 8 of Paper I

The two experiments A and B of Fig 2 are typical of the large majority and show the following sequence of events facilitation of a very modest order at the foot of the contraction (this effect was large in extensors) increasing into Phase II or quickly supplanted by inhibition while contraction increases (similar to the inhibition in the extensors) Early in the relaxation Phase III there may or may not be a hump of rising excitability (just as in extensors) but in the post contraction in Phase IV there is facilitation in the flexors In this phase the typical event in the extensor nuclei was inhibition, often profound

As always in experiments of this kind the central setting of the spinal cord is important and introduces variations Fig 2 C represents a case in which the central setting has favoured excitation so that the inhibitory effect during contraction is small The post contraction facilitation of Phase IV is always

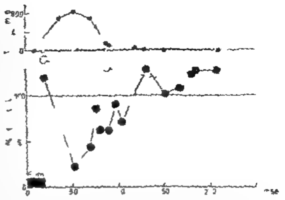

Fig 4 Time course of autogenetic changes of ankle flexor motoneurone excitability (monosynaptic reflex) following conditioning contraction before and after Flaxedil at intensity 8 times a threshold. Abscissae time in msec from commencement of conditioning stimulation as indicated by heavy line Ordinate lower curve amplitude of monosynaptic reflex expressed in per cent of its unconditioned amplitude upper curve tension in g ● before and ○ after Flaxedil

seen in such cases Finally Fig 2 D is an example of two experiments in which the post contraction facilitation was relative only. The contraction was small Nevertheless there was good inhibition which disappeared with the disappearance of tension

Phase I The facilitation which as a rule was smaller in flexors than in extensors began before the myogram displayed a rise of tension Occasionally as in Fig 3 it was separated from the facilitation in Phase II which is synchronous with the onset of measurable contraction Curarization by Flaxedil suffices to remove facilitation in Phase I (Fig 3) It did not remove it in the extensors

Phase II The inhibition during the rise of tension disappeared after Flaxedil as shown in Figs 3 and 4 and was replaced by facilitation when contraction was blocked Such facilitations lasted for a longer time in flexors than in extensors in fact during the whole of Phase III Increase of stimulus strength to 8 to 10 times the α threshold reduced the inhibition

Phase III More often than in extensors the maximum inhibition was slightly delayed with respect to maximum tension The transient facilitation which occurs in both extensors and flexors was in the former identified with the myotatic hump or appendage of Ballif Fulton and Liddell (1925) It is similarly interpreted in the flexors In both cases it is dependent upon natural spindle activation caused by the pull on the sense organ in relaxation It was absent after Flaxedil (Fig 4) when the tension changes had disappeared

The use of Flaxedil in our experiments is based on the fact that for some time after complete block of the α motor end plates the γ terminals still are capable of activating the intrafusal muscles as judged by the increased spindle discharge after strong tetani (Granit Homma and Matthews 1959) repeatedly confirmed in this laboratory and now again checked with flexor spindles The selective block is however more reliable with extensors in which the average dose of 2 mg/kg wholly removed contraction while the spindles still functioned practically undisturbed for some 15 to 30 min or more In flexors the margin between α and γ block is narrower The dose of 2 mg kg did not suffice for complete extrafusal block and with slightly larger doses the time available

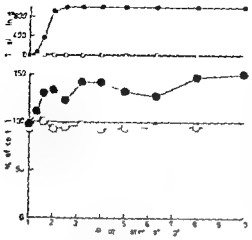

Fig 5 Relationship of conditioning stimulus strength to muscle contraction and motoneurone excitability Abscissae conditioning stimulus intensity as multiples of threshold for contraction. Ordinate upper curve tension in g at maximum of contraction lower curve amplitude of monosynaptic reflex in post-contraction phase (190 msec after commencement of conditioning stimulation) ● before and ○ after Flaxedil

for good spindle activation was *shortened to a few minutes only which means that the time course of flexor motoneurone excitability after Flaxedil had to be tested against a* background less constant than with extensors

Phase II In extensors the inhibition after relaxation did not appear unless the muscle actually had contracted and hence it was absent after Flaxedil There is a general similarity in this respect between the two antagonist motor nuclei in spite of the fact that Phase IV is excitatory in flexors and inhibitory in extensors After Flaxedil in Fig 4 facilitation diminished In Fig 5 it is seen that post-contraction facilitation as a function of stimulus strength runs parallel with the increase of the contraction and fails to be further increased by stimuli (the early tetani shown in the other experiments) reaching well beyond the threshold After Flaxedil the post-contraction facilitation is gone In every respect therefore the post-contraction changes of excitability in the two antagonist motor nuclei behaved as if similar events in the muscle receptors had acted in opposite direction on their executive organs the motoneurones

Discussion

In extensors there is a quite powerful rise of excitability with a latent period as short as 3 msec (Granit 1950) again confirmed in our Paper I Part of it may have been caused by the ephaptic component of stimulation the so-called back response (Lloyd 1941 1942 Leksell 1945) analyzed by Granit Pompeiano and Waltman (1959) in its dependence on tension and extension They found ephaptic excitation less prominent in the flexors used above than in the ankle extensors When now the facilitation in Phase I also is less prominent in flexors than in extensors it may well mean that ephaptic excitation is more important than our work on extensors suggested We were surprised there to find the facilitation of Phase I insensitive to Flaxedil because according to Lloyd (1942) curarization should depress ephaptic excitation

The early discharge from muscle receptors in contraction (the term being Hunt and Kuffler's 1951) was found to have a second component (Granit Pompeiano and Waltman 1959) synchronous with contraction difficult to distinguish from the ephaptic component in extensors but visible in flexors in which its existence also has been demonstrated in flexor reflexes (Rutledge and Haase 1961) This component of the early discharge has been ascribed *to a* spindle activation which since has been clearly demonstrated by direct recording in the cat's toe flexor (Bessou Emonet Denand and Laporte 1963) Presumably this is responsible for the early facilitation preceding the inhibition that further tension development engenders (Phase II)

Autogenetic inhibition dependent on tension in contraction is well known from work on extensors the relevant literature being referred to in our Paper I We found this effect to disappear after Flaxedil In this respect flexors and extensors behave similarly In the latter it has generally been identified with the Group I disynaptic inhibition of Laporte and Lloyd (1952) nowadays often called Ib and shown to contain in addition a number of polysynaptic links (Eccles Eccles and Lundberg 1957) Laporte and Lloyd found this type of inhibition also in flexors and so there is some reason for extending identification of Ib or tendon organ inhibition to the present finding in flexors in which it now has been shown to be autogenetic and tension dependent

The myotatic hump in Phase III has already been identified with the equivalent event in the extensors (above and in Paper I)

The finding which to us seems of greatest interest in the present work is the post contraction excitation in Phase IV It has already been pointed out that this event is a replica with opposite sign of a similar process in the extensor motoneurones in them ascribed to the effect of spindle secondaries It is not necessary here to repeat more than one argument for this identification namely the fact that secondaries are known to excite the flexor reflex (Hunt 1954 R M Eccles and Lundberg 1959 Laporte and Bessou 1959) which means that they are inhibitory for extensor nuclei and excitatory for flexor nuclei as required by our identification of the two post contraction events with opposite sign in the antagonist nuclei It follows that the changes of autogenetic excitability in Phase IV cannot be a consequence of engagement as such of motoneurones leading to refractoriness Such processes would be of identical sign in flexors and extensors

We have *no basis for evaluating the relative role of pre and postsynaptic* inhibitions in this paper (cf Eccles Eccles and Magni 1961) and have therefore restricted interpretation to the probable peripheral events in the muscle receptors Clearly however the experiments have provided a basis for an experimental identification of the inhibitory processes concerned

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Zur Wirkung subdiastolischer Okklusionsdrucke auf die distale Extremitätendurchblutung

Von

KLAUS GRAF und SUNE ROSELL

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Abstract

GRAF K. and S ROSELL, *Zur Wirkung subdiastolischer Okklusionsdrucke auf die distale Extremitatendurchblutung Acta physiol scand* 1964 *61* 357—375 — The effects of subdiastolic pressures in a proximal cuff on the arterial inflow rate into the human forearm or the hind leg of the cat were studied The arterial inflow was recorded simultaneously by venous occlusion plethysmography and by either thermal conductivity recorders or a drop recorder Subdiastolic cuff pressures which are usually used for venous occlusion in plethysmographic inflow measurements were found to reduce the arterial inflow The reduction was most pronounced initially During the first 10 sec the diminution of the arterial inflow was 4—6 per cent in forearm muscle and 20—30 per cent in the hind leg of the cat The plethysmographic inflow curve usually showed no corresponding upwards convex deflection but ascended rather steadily It is suggested that the initial reduction of the arterial inflow occurred instantaneously and was the effect of slight arterial narrowing caused by the cuff pressure In forearm muscle such subdiastolic pressure periods even as short as 5 sec were followed by slight reactive hyperaemia It is concluded that in the human upper arm the degree of arterial obstruction is relatively small and the phenomenon at least at moderate levels of blood flow therefore will not essentially invalidate the method of venous occlusion plethysmograph

Es ist haufig diskutiert worden ob die subdiastolischen Manschettendrucke die zu okklusionsplethysmografischen Durchblutungsmessungen verwandt werden auch die Messgrosse selbst den arteriellen Einstrom beeinflussen konnen (Dawes 1954 Greenfield et al 1963) Plethysmografisch wurde eine sichere Verminderung des arteriellen Einstromes erst dann gefunden wenn das Volumen des Messbereiches durch aufgestautes Blut um mindestens 2—3 % zugenommen hatte (Greenfield und Patterson 1954 Coles und Kidd 1957) Initial aber wahrend der Registrierung der plethysmografischen Einflusskurven zeigten plethysmografische und Druckmessungen keinen Anhalt fur eine solche Beeinflussung Vielmehr galt der anfänglich meist lineare Anstieg der Einflus-

kurven als Beweis für die Konstanz des arteriellen Einstromes (Greenfield 1957 und 1960) Und auch eine plotzliche initiale — von der Einflusskurve noch nicht angezeigte — Verminderung des arteriellen Einstromes auf ein neues konstantes Niveau wurde fur unwahrscheinlich gehalten weil subdiastolische Manschettendrucke den distalen arteriellen Druck noch nicht veranderten (Wilkins und Bradley 1946 Formel und Doyle 1957) Es wurde folglich angenommen dass plethysmografische Einflusskurven an Unterarm und Wade den gleichen arteriellen Einstrom anzeigten der auch ohne Beeinflussung der Extremitat gefunden worden ware (Greenfield et al 1963)

Indes mussen auch die ublichen Okklusionsdrucke von 50—80 mm Hg schon den effektiven Innendruck der arteriellen Gefasse (transmuralen Druck) vermindern, woraufhin diese — sofern sie elastisch und durch den Blutdruck gedehnt sind — wahrscheinlich ihr Lumen verengern Tatsachlich fanden Conrad und Green (1961) bei direkten Durchblutungsmessungen am Hundebein dass schon Manschettendrucke von 40 mm Hg den arteriellen Einstrom plotzlich um 7 % verminderten Grant und Pearson (1937) sahen dass am Oberarm Okklusionsdrucke von 50 mm Hg die plethysmografisch gemessene Unterarmdurchblutung erheblich reduzierten wenn die arteriellen Gefässe uberwiegend aus kleinkalibrigen Kollateralen bestanden Andere Autoren fanden dass der arterielle Einstrom in Unterarm Hand und Fuss mit steigendem Okklusionsdruck abnahm (cf Graf und Rosell 1964)

Zur weiteren Untersuchung des Einflusses subdiastolischer Okklusionsdrucke wurde der arterielle Einstrom in die Hinterextremitat der Katze und in den menschlichen Unterarm plethysmografisch sowie gleichzeitig auch mit einem zweiten Verfahren (Tropfenmessung Warmeleitmessung) registriert Auch der lokale arterielle Druck wurde bestimmt Es zeigte sich dass subdiastolische (venose) Okklusionsdrucke wie sie bei der Okklusionsplethysmografie verwandt werden auch den arteriellen Einstrom initial geringfugig vermindern Diese initiale Verminderung war weder aus dem Verlauf der plethysmografischen Einflusskurve zu erkennen noch fuhrte sie zu messbaren Anderungen des arteriellen Druckes Grosse und Verhalten dieser Beeinflussung des arteriellen Einstromes und ihre Bedeutung fur okklusionsplethysmografische Durchblutungsmessungen wurden naher untersucht

Methodik

Messungen an der Hinterextremitat der Katze

Die Versuche erfolgten an 3 Katzen (Gewicht 1 9 bis 2 8 kg) Anasthesie mit Chloralose (50 mg/kg i v) plus Urethan (0 1—0 2 g/kg i v) Trachealkanulierung Die Rektaltemperatur wurde durch Bestrahlung mit einer Warmelampe auf 36—37 C gehalten Blutdruckmessung in der A carotis dx mit Elektromanometer Statham P 23 AA Die A. femoralis dx wurde im unteren Drittel des Oberschenkels ligiert und die arterielle Durchblutung zum Unterschenkel durch einen fotoelektrischen Tropfenzähler geleitet der einen Ordinatenschreiber steuerte (Landgren 1958) (Fig 1) Jede unnötige Ab-

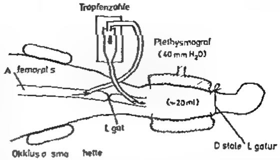

Fig. 1. Versuchsanordnung zur gleichzeitigen direkten und plethysmografischen Messung des arteriellen Einstromes an der Hinterextremität der Katze. D. Grenzen des ca. 20 ml grossen plethysmografischen Messbereiches sind durch unterbrochene Linien bezeichnet

lösung der Haut von der Muskulatur wurde bei der Präparation sorgfältig vermieden. Die *Registrierung von Durchblutung, arteriellem Druck, Zeit* (1 min) und Markierungssignalen erfolgte mit einem Grass Polygraphen (Registrierbeispiel in Fig. 2). Der arterielle Einstrom in den rechten Unterschenkel wurde mit einem luftgefüllten (Füllungsdruck 40 mm H_2O) flexiblen Dohn'schen Segmentplethysmografen gemessen (Fig. 1). Der 5 cm lange Plethysmograf umschloss die Wade in ihrer ganzen Länge. 1 cm distal *vom Plethysmografen wurde die Durchblutung durch eine Ligatur unterbunden*. Das Volumen des Bereiches für die direkte Registrierung des arteriellen Einstromes hatte eine Grösse von durchschnittlich 27 (25—30) ml, während sich die plethysmografischen Messungen auf einen um 28 (13—44) % kleineren Bereich von 20 (14—26) ml Grösse bezogen. Okklusionen zur plethysmografischen Durchblutungsmessung erfolgten in *Oberschenkelmitte 3—5 cm proximal vom Abgang der zum Tropfenzähler führenden* Femoraliskanüle (Fig. 1). Die Okklusionsmanschette bestand aus dünner Plastikfolie und hatte eine Breite von 2—3 cm. Ihr Füllungsdruck wurde mit einem Elektromanometer (Elema) gemessen. Eine Binde verhinderte ihr Verrutschen nach distal hin. Die plethysmografischen Messungen erfolgten mit der von Graf und Westersten (1959) beschriebenen *Apparatur. Plethysmogramm und Okklusionsdruck wurden mit einem* 4-Kanal-Direktschreiber registriert (Mingograf plus Elektromanometer 42, Elema, Stockholm-Solna) (Registrierbeispiel in Fig. 2); die lineare Schreibbreite betrug für jeden Kanal 6—8 cm.

Bei den 3 Katzen erfolgten 82 Okklusionen mit subdiastolischen Manschettendrücken *bis zu 80 mm Hg und 32 Okklusionen mit höheren Drücken*.

Messungen am Unterarm

Die Versuche erfolgten an 16 gesunden, liegenden Personen (Studenten). Die (direkt oder auskultatorisch gemessene) Blutdruckamplitude in der A. brachialis dx. lag zwischen 140 und 65 mm Hg. Störungen durch die Umgebung oder unbequeme Lagerung wurden sorgfältig vermieden. Zimmertemperatur 23 °C.

Durchblutungsmessungen mit venöser Okklusionsplethysmografie erfolgten am proximalen Teil des rechten Unterarmes mit 5 cm langen, luftgefüllten, flexiblen Segmentplethysmografen nach Dohn (Einzelheiten bei Graf und Westersten 1959). Der Unterarm war in *einer Höhe von ca. 5 cm über dem Niveau des Manubrium sterni gelagert*. Die Staumanschette sass am Oberarm unmittelbar proximal vom Ellenbogen. 1—2 cm distal vom Plethysmografen war eine Manschette angebracht, um während der Durchblutungsmessungen den venösen Rückfluss in das Messgebiet zu blockieren. Die beiden Okklusionsmanschetten wurden gewöhnlich synchron auf den gleichen Druck aufgeblasen, die distale Manschette *jedoch auf nie weniger als 70 mm Hg. Weiteres bei Graf (1964* a und b).

Bei 5 Personen erfolgten am selben (rechten) Arm auch Messungen der Handdurchblutung mit flexiblen luftgefullten Endplethysmografen nach Dohn (Einzelheiten bei Graf und Westersten 1959) Okkludiert wurde mit der Unterarmmanschette die distal vom Segmentplethysmografen sass

Durchblutungsmessungen mit Warmetransportzahlmessern (WTM) nach Hensel erfolgten gleichzeitig mit den plethysmografischen Durchblutungsregistrierungen und am selben Arm bei 10 Personen Die WTM wurden im plethysmografischen Messbereich am Unterarm plaziert der Muskel WTM (Sonde) in die Brachioradialis Extensorenmuskulatur eingefuhrt der Haut WTM (Platte) unter die innere Lamelle des Plethysmografen geschoben Die Plazierung war identisch mit der schematischen Darstellung in Fig 1 bei *Graf (1964 a)* Weiteres zur Registrierung und Auswertung von Warmetransportzahlmessungen bei Golenhofen et al (1963)

Eine Kalibration der Warmeleitmessung in Werten der Warmeleitzahlerhohung $\Delta\lambda$ (10^{-4}cal/cm s C) halt Bill (1962) nicht fur sinnvoll Er bestatigte in Modellversuchen dass die Proportionalitat zwischen Anderungen von $\Delta\lambda$ und Durchflussvolumen bei hoheren lokalen Durchflusswerten immer geringer und $\Delta\lambda$ mit abnehmendem Abstand zwischen Messort und Gefassen sichtbarer Grosse immer grosser wird wodurch quantitative Messungen schwierig werden Er liess jedoch unerwahnt dass die Tragheit der Anzeige von Durchflussanderungen mit steigenden Werten von $\Delta\lambda$ geringer wird was bei der kontinuierlichen Registrierung von Durchblutungsanderungen von erheblicher Bedeutung sein kann Zur Beurteilung der Anzeigeverzogerung von Durchblutungsanderungen konnen daher auch Angaben uber die Grosse von $\Delta\lambda$ erforderlich sein (Graf und Rosell 1958)

Die Disproportionalitat zwischen den Anderungen von $\Delta\lambda$ und Durchflussvolumen lasst Bill (1962) auch eine semiquantitative Kalibration der lokalen Durchblutungsanderungen ablehnen *sofern nicht jede WTM Lage mit direkter Durchflussbestimmung* ... wird Eine grossere Zahl von Untersuchungen hat jedoch gezeigt dass im physiologischen Durchblutungsbereich das Verhaltnis zwischen Anderungen von $\Delta\lambda$ und Durchflussvolumen meist noch weitgehend linear ist (Literatur bei Golenhofen et al 1963)

Arteriennahe Sondenlagen Zur Erfassung des arteriellen Einstromes im Muskel wurden moglichst arteriennahe Sondenlagen aufgesucht Da die grosseren Arterien haufig eng zusammen mit ihren zugehorigen Venen verlaufen sind aber meist nur Sondenlagen mit einer nur uberwiegenden Anzeige der arteriellen Stromung zu erhalten (siehe auch Golenhofen et al 1963) Die folgenden 3 Kriterien fur eine arterielle Registrierung erfullte bei Messungen in den Unterarmextensoren im Durchschnitt jede 15 Sondenlage (sonst war dort jede 5 bis 8 Sondenlage brauchbar) *1)* pulsatorische Durchblutungsschwankungen *2)* eine durchblutungsbedingte Warmetransportzahlerhohung von mindestens 1 5 10^{-4}cal/cm s C *3* eine Erwarmung der ungeheizten Messlotstelle bei reaktiver Hyperamie Eine Besprechung weiterer Besonderheiten arteriennaher Sondenlagen siehe in Diskussion

Druckmessungen Bei 8 Personen wurde zusatzlich zur Durchblutung im rechten Unterarm auch der Druck in der A brachialis dx uber einen perkutan eingefuhrten Katheter mit einem Elektromanometer (Elema) fortlaufend gemessen Die Katheterspitze lag in Hohe der Fossa cubiti 2—3 cm vom distalen Rand der Oberarm-Okklusion manschette entfernt (entsprechend Fig 1 bei Graf 1964 a)

Registrierungen Plethysmogramm und arterieller Druck wurden gleichzeitig auf einem 4-Kanal Direktschreiber (Mingograf plus Elektromanometer 42 Elema Stockholm Solna) registriert (Registrierbeispiele in Fig 5 und 6) die lineare Schreibbreite betrug fur jeden Kanal 6—8 cm Die Warmetransportzahlregistrierungen erfolgten auf einem 2 Kanal Direktschreiber (Fluvograph Hartmann & Braun Frankfurt)

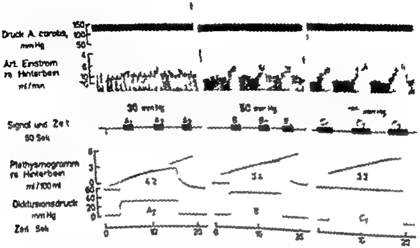

Fig. 2 Abschnitte direkter und plethysmografischer [illegible] des arteriellen Einstromes an der Hinterextremität einer Katze bei Okklusionsdrucken [illegible] mm Hg. Obere 3 Kurven: Registrierungen mit Grass-Polygraph (beach[illegible] Ordinatenschreibung). Signal für Okklusionsdauer. Untere 2 Kurven: [illegible] Druckstufe wird aus Platzgründen nur eine plethysmografi[illegible] Für jede und C_1 synchron zu den direkten Einstrommessungen [illegible] den Einflusskurven. Angabe der plethysmografisch gemessenen Durch[illegible] Unter Grösse des plethysmografischen Messbereiches [illegible]

Ergebnisse

Messungen an der Hinterextremität der Katze

Direkte Messungen des Einstromes durch die A. fem[illegible] Schon Okklusionsdrucke von 20 mm Hg verminderten häufig die D[illegible] der A. femoralis. Mit zunehmendem Okklusionsdruck wurde diese Einstromverminderung stärker (Fig. 2). Bei Okklusionen mit 40 mm Hg war die Abnahme des arteriellen [illegible] stromes in allen Fällen sicher nachweisbar und [illegible] anderen Schwankungen [illegible] arteriellen Einstromes deutlich zu unterscheiden. Bei gleichem Okklusion[illegible] waren die Verminderungen des arteriellen Ei[illegible] umso stärker [illegible] der Umfang des Oberschenkels unter der Okklusionsmanschette [illegible] Okklusionsdrucke von 140–180 mm Hg drosselten die A. femoralis vollstä[illegible] einem Druck in der A. carotis von etwa 160 [illegible] mm Hg).

Der Druck in der Okklusionsmanschette [illegible] sich in weniger [illegible] konstant ein [illegible] Die Abnahme des arteriellen Einstromes [illegible] der Haupt[illegible] aber [illegible] ersten 10 sec nach Okklusionsbeginn [illegible] wurden 60–80 [illegible] dieses Abfalles gew[illegible] unmittelbar nach Okkl[illegible]

Tab I Ergebnisse gleichzeitiger direkter und plethysmografischer Messungen des arteriellen Einstromes an derselben Hinterextremität der Katze bei verschiedenen subdiastolischen Okklusionsdrucken. Werte aus 44 Messungen an 3 Katzen Die direkten Einstrommessungen erfolgten jeweils nach 10 sec Okklusionsdauer Abnahme des arteriellen Einstromes in % der Ausgangswerte Die in Parenthese stehenden plethysmografischen Werte bei 0 mm Hg wurden — entsprechend der direkten Einstromdifferenz — von den bei 20 mm Hg gemessenen Werten aus extrapoliert Mittelwerte (M) und Bereiche (r)

		Okklusionsdruck (mm Hg)				
		0	20	40	60	80
Arterieller Einstrom (ml/min)						
Tropfenmessung	M	*3.3*	*3.2*	*3.0*	*2.5*	*2.3*
	r	1.0–4.9	0.92–4.8	0.86–4.7	0.65–3.9	0.42–3.7
Plethysmografische Messung	M	[*2.5*	*2.4*	*2.1*	*1.9*	*1.8*
	r	0.91–3.8]	0.82–3.7	0.70–3.2	0.67–2.8	0.58–2.8
Abnahme des arteriellen Einstromes während der Okklusion (%)						
Tropfenmessung	M	*0*	*−4*	*−11*	*−26*	*−34*
	r		−(0−10)	−(4−20)	−(15−40)	−(16−60)
Plethysmografische Messung	M	*0*	*−4*	−17	−25	*−31*
	r		−(0−10)	−(4−23)	−(16−32)	−(16−34)

beginn registriert (Fig 2 beachte die nicht vertikale Ordinatenschreibung und die nicht lineare Registrierung des arteriellen Einstromes)

Tab I zeigt die Verminderungen des arteriellen Einstromes bei Okklusionen mit 20—80 mm Hg Okklusionsdrucke von 20—40 mm Hg mit denen an der Katze die besten (steilsten) Einflusskurven registriert wurden verminderten den arteriellen Einstrom während der plethysmografischen Messdauer von 10 sec bereits um 4—11 %

Bei 18 Messungen wurden die Okklusionen mit subdiastolischen Drucken bis zu 2 min ausgedehnt. Verglichen mit der Einstromverminderung in den ersten 10 sec fiel der arterielle Einstrom dabei nur noch geringfügig weiter ab So betrugen bei 60 mm Hg Okklusionsdruck die Abnahmen nach 2 min 20—60 % bei 80 mm Hg 20—80 % (vergleiche die Werte für die Einstromverminderungen nach 10 sec Okklusionsdauer Tab I) Bei einem Versuch wurde nach 30—60 sec Okklusion mit 20—40 mm Hg zweimal ein geringer eben angedeuteter sekundärer Wiederanstieg des arteriellen Einstromes beobachtet der jedoch den Ausgangswert nicht überschritt und schon in der nächsten Minute wieder zurückging

Plethysmografische Messungen Die plethysmografischen Einflusskurven vom Unterschenkel liegen in den ersten 10 sec gewöhnlich linear an (Fig 2) und

Tab II Durchblutungsabnahme und nachfolgende reaktive Hyperämie in der Unterarmmuskulatur bei 5—120 sec langen Okklusionsperioden am Oberarm mit 70 mm Hg Messungen mit der Wärmeleitsonde an 8 Personen Bezugswerte für Durchblutungsabnahmen und Flächenwerte waren die Änderungen nach 5 min kompletter Durchblutungsdrosselung mit 250 mm Hg Mittelwerte (M) und Bereiche (r)

		Okklusion mit 70 mm Hg (Dauer in sec) 5	10	20	30	60	120	Okklusion mit 250 mm Hg (5 min Dauer)
Durchblutungsabnahme während der Okklusion (% des mittleren Ausgangswertes)	M	*−4*	*−6*	*−10*	*−15*	*−14*	*−17*	*−100*
	r	−(1–8)	−(4–9)	−(8–13)	−(12–19)	−(13–17)	−(14–19)	
Durchblutungszunahme im Maximum der reaktiven Hyperämie (% des mittleren Ausgangswertes)	M	*+5*	*+11*	*+29*	*+67*	*+146*	*+256*	*+398*
	r	0—8	7—14	19—38	51—100	128—175	270—292	350—470
Flächenwert der reaktiven Hyperämie ( )	M	*0.3*	*0.7*	*1.8*	*4.5*	*14.8*	*37.6*	100
	r	0.1—0.4	0.3—1.4	1.0—2.9	1.8—6.8	7.3—22.8	28.6—46.0	

liessen also weder einen plötzlichen initialen Abfall noch eine mehr allmählich erfolgende Verminderung des arteriellen Einstromes erkennen Bei höheren Okklusionsdrucken mit stärkerer initialer Verminderung des arteriellen Einstromes war der Anstieg der Einflusskurven oft länger linear als bei geringeren Okklusionsdrucken (Fig 2)

Vergleich von direkt und plethysmografisch gemessenen Werten Die plethysmografisch gemessenen Werte des arteriellen Einstromes waren im Durchschnitt um 20—35 % geringer als die direkt gemessenen (Tab I) Diese Differenz entsprach gut dem Grössenunterschied der beiden Messbereiche (Werte in Methodik) Hingegen wurden die relativen Verminderungen des arteriellen Einstromes durch die Okklusionsdrucke von beiden Verfahren ähnlich angegeben (Tab I)

Reaktive Hyperämie Okklusionen mit Drucken bis zu 80 mm Hg und einer Dauer bis zu 20 sec waren von keiner oder einer nur schwach angedeuteten reaktiven Hyperämie gefolgt (Fig 2) Eine geringe und nicht länger als 20 sec anhaltende reaktive Hyperämie erfolgte hier erst nach einer Okklusionsdauer

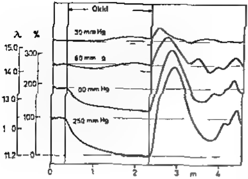

Fig 3 Verhalten der Unterarmdurchblutung (arterieller Einstrom) bei verschieden starken Okklusionen am Oberarm Kurven aus einem Versuch in der gezeigten Reihenfolge registriert Kalibration in λ (10^{-4}cal/cm s C) und % des mittleren Ausgangswertes (λ und % Skalen sind hier nur der untersten *Registrierung zugeordnet für die anderen* Kurven müssen sie bis zu deren Ausgangswerten parallel verschoben werden) Die gestrichelten Linien in den oberen Kurven bezeichnen den in anderen Versuchen bei Okklusionsdrucken *von 30—60 mm Hg häufiger registrier*ten Durchblutungsverlauf

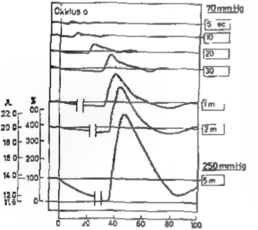

Fig 4 Verhalten der Unterarmdurchblutung (arterieller Einstrom) bei 5—120 sec langen Okklusionen am Oberarm mit 70 mm Hg Kurven aus einem Versuch in der gezeigten Reihenfolge registriert Zeichnerische Unterbrechung des kontinuierlichen Kurvenabfalles in den 3 unteren Kurven Schraffierte Bereiche über der 100 Linie Flächenwerte der reaktiven Hyperämie Kalibration in λ (10^{-4}cal/cm s C) und des mittleren Ausgangswertes (λ und % Skalen sind hier nur der untersten Registrierung zugeordnet für die anderen Kurven müssen sie bis zu deren Ausgangswerten parallel verschoben werden) Vergleichswerte sind die Reaktionen bei 5 min langer kompletter Durchblutungsdrosselung mit 250 mm Hg

von 1—2 min Dagegen zeigten in denselben Versuchen Kontrollen mit 20 sec vollständiger Drosselung des arteriellen Einstromes durch Okklusion mit 250 mm Hg *eine deutliche reaktive Hyperämie von etwa 15 sec Dauer* deren Ausmass (Flächenwert) bei längeren Okklusionen in linearer Abhängigkeit zur Okklusionsdauer (gemessen bis zu 2 min) zunahm

Messungen im Unterarm

Durchblutung (arterieller Einstrom) im Muskel In der Unterarmmuskulatur führten schon Okklusionsdrucke am Oberarm von 30 mm Hg zu einer Verminderung des arteriellen Einstromes Mit zunehmendem Okklusionsdruck wurde *diese Verminderung immer stärker und erreichte schliesslich* bei kompletter arterieller Drosselung durch 250 mm Hg den Durchblutungs Nullwert (*Tab II* Fig 3) Die arterielle Einstromverminderung war in den ersten 10 sec der Okklusion am stärksten setzte aber gewöhnlich auch in den anschliessenden 2 min noch langsam weiter *fort (Tab II Fig 3 und 4)* Bei 70 mm Hg Okklusionsdruck betrug die arterielle Einstromverminderung in den ersten 10 sec — also

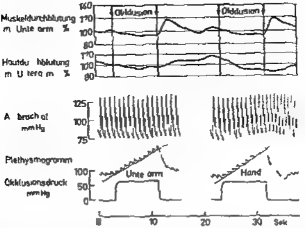

Fig. 5. Gleichzeitige Registrierungen von Durchblutung am rechten Unterarm und in Muskulatur und Haut des plethysmografischen Messbereiches sowie der Drucke in der A. brachialis dx. und der Okklusionsmanschette am Oberarm. Die Registrierungen erfolgten mit gleicher Papiergeschwindigkeit. Die 2. Einflusskurve wurde nach Umschaltung des Plethysmografen an der Hand registriert. Muskel- und Hautdurchblutung in % des mittleren Ausgangswertes. Durchblutung im Unterarm 4,1 ml/100 ml · min, in der Hand 3,4 ml/100 ml · min.

in der zur Registrierung plethysmografischer Einflusskurven benötigten Zeit — durchschnittlich 6 % (Tab. II) — die wirkliche Einstromverminderung dürfte in dieser Zeit noch etwas grösser und initial noch steiler gewesen sein, was aber infolge der Trägheit der Wärmetransportzahlmessung nicht vollständig angezeigt wurde.

Okklusionen mit subdiastolischen Drucken, auch schon von nur 5 oder 10 sec Dauer, reduzierten oft die spontan rhythmischen Schwankungen der Muskeldurchblutung im 30—60 sec Rhythmus oder konnten diese auch ganz aufhören lassen (Fig. 3). Nach Okklusionsende verging bis zu ihrer vollständigen Rückkehr häufig 1 min.

Bei 3 Personen wurde im Anschluss an den initialen Durchblutungsabfall mehrfach (bei unveränderter Sondenlage) ein sekundärer, vorübergehender Wiederanstieg der Wärmetransportzahlregistrierung bis über den Ausgangswert beobachtet. Dieser Wiederanstieg begann 30—60 sec nach Okklusionsbeginn, ging aber schon in der nächsten Minute wieder allmählich zurück. Ein Beispiel dafür zeigt Fig. 3 (ausgezogene Linien). Auch der leichte Anstieg des Mittelwertes der arteriellen Einstromverminderung nach 60 sec Okklusion in Tab. II deutet dieses Verhalten an. Diese Reaktion erfolgte nur bei Okklusionen mit Drucken bis zu 80 mm Hg; höhere Drucke bewirkten auch bei diesen Versuchen nur einen kontinuierlichen Kurvenabfall. In allen anderen Versuchen erfolgte auch bei Okklusionen mit geringeren Drucken als 70 mm Hg nur ein kontinuierlicher Abfall des arteriellen Einstromes (angedeutet durch die unterbrochenen Linien der beiden oberen Kurven der Fig. 3), dieses war auch

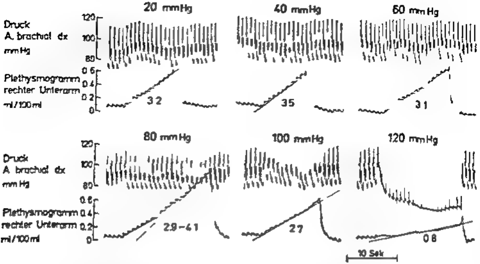

Fig 6 Plethysmografische Einflusskurven am rechten Unterarm und Druck in der A brachialis dx. bei steigenden Okklusionsdrucken am Oberarm. Angabe des Okklusionsdruckes uber den Druckkurven Kurven aus einem Versuch in der gezeigten Reigenfolg registriert Die Abstande zwischen den Okklus onen betrugen mindestens 10 sec Weiteres im Text

der oben erwahnten 3 Personen der Fall wenn mit einer anderen Sonderlage gemessen wurde

Hautdurchblutung Okklusionen mit 50 mm Hg und mehr fuhrten auch zu einer Abnahme der Hautdurchblutung am Unterarm Diese war initial am starksten und konnte in den ersten 10—15 sec 10 % und mehr betragen (Fig 5) Ein sekundarer Wiederanstieg wie im Muskel (Fig 3) wurde nicht beobachtet Die wirklichen Abnahmen der Hautdurchblutung durften besonders initial erheblich grosser und steiler gewesen sein was aber infolge der Anzeigetragheit dieser Warmeleitzahlmessungen unvollstandig registriert wurde damit erklart sich auch die Verzogerung gegenuber den gleichzeitig registrierten Reaktionen der Muskeldurchblutung (Fig 5)

Plethysmografische Messungen der Unterarmdurchblutung Die initiale Verminderung des arteriellen Einstromes um 5—10 % die in den ersten 10 sec in Muskulatur und Haut des Unterarmes erfolgte wurde von gleichzeitig registrierten plethysmografischen Einflusskurven nicht angezeigt diese stiegen vielmehr meist linear an (Fig 5)

Die steilsten Einflusskurven wurden bei Okklusionsdrucken von 40—70 mm Hg registriert Bei hoheren aber noch subdiastolischen Okklusionsdrucken wurden die Einflusskurven allmahlich flacher Bei Okklusionsdrucken die zwischen der Grosse des diastolischen Druckes und der des arteriellen Mitteldruckes lagen konnten die Einflusskurven — trotz Verminderung des arteriellen Mitteldrukkes — noch einmal signifikant ansteigen (Fig 6) Bei noch hoheren Okklusionsdrucken wurden die Einflusskurven rasch flacher (Fig 6)

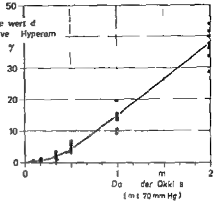

Fig 7 Flachenwerte der reaktiven Hyperamie in der Unterarmmuskulatur nach 5—120 sec langen Okklusionen am Oberarm mit 70 mm Hg Messungen mit der Wärmeleitsonde an 8 Personen 100 = Flachenwert nach 5 min kompletter Durchblutungsdrosselung mit 250 mm Hg Ausgemessen wurden nur die Flachen der ersten Durchblutungszunahmen die die 100 % Linie uberstiegen (schraffiert gezeichnet in Fig 4)

Druck in der A brachialis Subdiastolische Okklusionsdrucke am Oberarm fuhrten zu keinen messbaren Veranderungen des Druckes im distalen Teil der A brachialis Okklusionsdrucke die zwischen der Grosse des diastolischen Druckes und des arteriellen Mitteldruckes lagen verminderten die systolischen und mittleren Druckwerte leicht Okklusionsdrucke die den arteriellen Mitteldruck uberstiegen reduzierten den gesamten distalen arteriellen Druck Siehe Beispiele in Fig 5 und 6

Reaktive Hyperamie Schon 5 sec lange Okklusionen am Oberarm mit 50—70 mm Hg hatten in der Unterarmmuskulatur eine reaktive Hyperamie zur Folge die mit zunehmender Okklusionsdauer und steigendem Okklusionsdruck starker wurde (Tab II Fig 3 und 4) In der Haut des Unterarmes war dabei eine deutliche reaktive Hyperamie nicht zu erkennen Im Muskel war die Grosse der reaktiven Hyperamie — der Flachenwert der ersten Durchblutungszunahme die die 100 % Linie uberstieg (schraffierte Flachen in Fig 4) — nach 5—10 sec langen Okklusionen noch minimal Nach 20 sec langen Okklusionen wurde der Flachenwert grosser und nahm von da ab in linearer Abhangigkeit zur Okklusionsdauer zu (Tab II Fig 7) Nach Okklusionen von weniger als 30 sec Dauer bestand die reaktive Hyperamie gewohnlich nur aus einer einmaligen Durchblutungszunahme Nach langeren Okklusionen erfolgte die Wiedereinstellung der Durchblutung haufig in Form von 2 bis 3 periodisch gedampften Schwingungen (angedeutet in Fig 3)

Diskussion

Initiale Abnahme des arteriellen Einstroms

Conrad und Green (1961) fanden in direkten Messungen an der Hundeextremitat schon bei Okklusionen mit 40 mm Hg plotzliche initiale Abnahmen des arteriellen Einstromes um durchschnittlich 7 % Golenhofen und Hildebrandt (1957, 1962) sahen bei Okklusionen am Oberschenkel mit 40 mm Hg

eine rasche initiale Abnahme der Muskeldurchblutung in der Wade. Greenfield und Patterson (1954) zeigten in ihrer Fig. 4 schematisch eine initiale, während der Registrierung der plethysmografischen Einflusskurven erfolgende Verminderung des „actual arterial inflow" von ca. 0,2—0,8 ml/100 ml min. Legt man dem die üblichen Ruhedurchblutungswerte im Unterarm von 2—5 ml/100 ml min zugrunde, so entsprach dieses einer initialen Verminderung des arteriellen Einstromes um 4—10 %, war also ähnlich den von Conrad und Green (1961) sowie den in diesen Untersuchungen gefundenen Durchblutungsabnahmen. Diese plötzlichen initialen Verminderungen des initialen Einstromes sind wahrscheinlich auf eine partielle Okklusion (Verengerung) der arteriellen Gefässe durch den subdiastolischen Okklusionsdruck zurückzuführen (siehe weiter unten unter „Bedeutung der arteriellen Einstromveränderungen für okklusions-plethysmografische Durchblutungsmessungen").

Offenbar sind die Abnahmen des arteriellen Einstromes umso stärker, je geringer Extremitätenvolumen und Arterienkaliber an der Okklusionsstelle sind. So war bei gleichen Okklusionsdrucken die relative Verminderung des arteriellen Einstromes stärker am Katzenbein als am Arm, und auch an den 3 Katzenbeinen war bei gleichen Okklusionsdrucken eine umgekehrte Proportionalität zwischen Einstromverminderung und Oberschenkelvolumen vorhanden. Diese Ergebnisse passen zu den Beobachtungen von Burch (1954) und Shanks (1955), die durch subdiastolische Okklusionsdrucke am Finger erheblich stärkere Durchblutungsabnahmen fanden als sie bei gleich starken Okklusionen an Unterarm oder Wade vorkommen. Zur Erklärung kann eine zunehmende radiäre Verminderung des effektiven Okklusionsdruckes angenommen werden, wodurch bei gleichem Manschettendruck die Arterien in voluminösen Extremitätenabschnitten (Oberarm, Oberschenkel) geringeren effektiven Okklusionsdrucken ausgesetzt werden als in schmaleren Segmenten (Finger, Katzenbein) (cf. Graf 1964 a).

Grant und Pearson (1937) fanden eine stärkere Abnahme des arteriellen Einstromes auch an kleineren Arterien (Kollateralgefässen). Arterien mit kleinerem Kaliber können elastisch dehnbarer und folglich stärker druckpassiv erweitert sein als grössere, weshalb ihr Lumen bei Verminderung des transmuralen Druckes relativ stärker abnehmen kann als das grösserer Arterien mit höherem Elastizitätsmodul (cf. Janeway und Park 1910, Mackay und Walker 1963).

Man könnte vermuten, dass der arterielle Einstrom in linearer Abhängigkeit vom ansteigenden Okklusionsdruck abnimmt, und solche Befunde sind auch vereinzelt erhoben worden (cf. Graf und Rosell 1964). Häufig wurden dagegen, wie in diesen Untersuchungen, bei ansteigenden subdiastolischen Okklusionsdrucken erheblich geringere Abnahmen des arteriellen Einstromes beobachtet. Die viel zitierten Untersuchungen von Landowne und Katz *(1942) sind sogar so gedeutet worden, dass subdiastolische* Okklusionsdrucke noch zu überhaupt keiner Verminderung des arteriellen Einstromes führen, indes zeigt Fig. [illegible] bei Landowne und Katz (1942) bei ansteigenden Okklusionsdrucken zwischen 50 und 120 mm Hg ebenfalls eine, wenn auch geringe, zunehmende Verminderung der in der Wade plethysmografisch gemessenen Durchblutungswerte. *In der* Nähe diastolischer Okklusionsdrucke ist sogar ein nochmaliger kurzzeitiger Anstieg des

arteriellen Einstromes möglich (Fig. 6). Wilkins und Bradley (1946) und Mackay (1957) erklären den bei subdiastolischen Okklusionsdrucken unproportional geringen Abfall und eventuell sogar nochmaligen Wiederanstieg des arteriellen Einstromes mit einem funktionellen „Klappeneffekt", der nur bei deutlich pulsatorischem Ablauf des arteriellen Einstromes auftrete: in der diastolischen Phase abnehmenden arteriellen Druckes würden die Arterien durch subdiastolische Drucke stärker okkludiert, wodurch ein pulsatorischer arterieller Rückfluss vermindert und das arterielle Netto-Einstromvolumen sogar erhöht werden könne.

Sekundäre Änderungen des arteriellen Einstromes

Im Anschluss an die rasche initiale Verminderung fiel der arterielle Einstrom nur langsam weiter ab. Die Ursache kann eine durch die Gefässauffüllung bedingte Erweiterung der Widerstandsgefässe sein. Eine solche ist häufig diskutiert und nach Ansteigen des Venendruckes um 10—12 mm Hg auch sicher gefunden worden (Linton et al. 1941; Friedland et al. 1943; Edholm et al. 1954; Greenfield und Patterson 1954; Moreira et al. 1956; Prerovsky und Linhart 1956; Formel und Doyle 1957; cf. Folkow 1962; Shepherd 1963).

Ein sekundärer Wiederanstieg des arteriellen Einstromes (Fig. 3) wurde ähnlich wie hier auch schon früher beobachtet (Pritchard et al. 1942; Golenhofen und Hildebrandt 1957; Schoop 1960 und 1961). Auch die angezweifelten Befunde von Linton et al. (1941) können eine ähnliche Reaktion angegeben haben. Grösse und Dauer der sekundären Durchblutungszunahme wurden mit steigendem Okklusionsdruck geringer, womöglich infolge der bei höheren Okklusionsdrucken zunehmenden Verminderung des arteriellen Einstromes. Die baldige Wiederabnahme des sekundären Durchblutungsanstieges ist mit dem mit zunehmender Gefässauffüllung verbundenen Anstieg des arteriellen Strömungswiderstandes zu erklären. Bei niedrigen Okklusionsdrucken von 20—40 mm Hg kann sich der Rückgang der sekundären Durchblutungssteigerung über mehrere Minuten hinziehen (Golenhofen und Hildebrandt 1957; Schoop 1961), weil die Auffüllung der Gefässe dabei langsamer erfolgt und auch geringer ist. Erst nach Volumenzunahmen durch aufgestautes Blut von 2—3 % nimmt der arterielle Einstrom regelmässig stärker ab (Greenfield und Patterson 1954; Coles und Kidd 1957).

Die Ursache dieser sekundären Durchblutungssteigerung ist unklar. Auffälligerweise wurde sie bisher nur in tiefen (Muskel-) Gefässen beobachtet, aber auch dort nicht regelmässig, was auf besondere Umstände hinweisen kann. Golenhofen und Hildebrandt (1957) halten es für möglich, dass dieser Durchblutungsanstieg nur in Abschnitten mit myogen reagiblen (tonisierten) Widerstandsgefässen erfolge, die also auf Abnahmen des transmuralen Druckes mit einer Dilatation reagieren können. Eine weitere Erklärungsmöglichkeit wäre ein Shiften der Durchblutung von superfiziellen (Haut) in die tieferen (Muskel-Knochen) Gefässe. Eine Reihe von Untersuchungen haben das Vorkommen einer solchen Reaktion wahrscheinlich gemacht, die frühestens 5—10 sec nach

Beginn venöser Okklusion auftreten, druckpassiv (oder auch reflektorisch) durch den Venendruckanstieg ausgelöst, und mit einer Zunahme des venösen Abstromes durch die tiefen Gefässe in Muskel und Knochen und gleichzeitiger Konstanz oder auch Abnahme der Hautdurchblutung verbunden sein soll (Moreira et al 1956, Petrovsky und Linhart 1956 Coles et al 1958, Shaw 1963)

Reaktive Hyperämie

Die reaktive Hyperämie nach subdiastolischen Okklusionen gibt wahrscheinlich nicht nur den Abfluss des aufgestauten Blutes an weil dieser wie plethysmografische Registrierungen zeigen schon nach 2—5 sec beendet ist Vielmehr war die reaktive Hyperämie nach subdiastolischen Okklusionen in Ausmass wie Dauer *proportional zu Länge und Grösse der Okklusionen was auch andere* Autoren mit verschiedenen Methoden an den Extremitäten gefunden hatten (Lewis und Grant 1925 Friedland et al 1943 Abramson 1946 Folkow 1949 Patterson und Shepherd 1954 Golenhofen und Hildebrandt 1962)

Auch nach 5 oder 10 sec langen venösen Okklusionen kam es schon zu einer reaktiven Hyperämie Nach so kurzen Okklusionen wird deren Ursache weniger in lokal chemischen Faktoren als mehr in einer druckpassiven Weiterstellung oder auch myogen bedingten Tonusabnahme der Widerstandsgefässe gesehen (cf Folkow 1949 und 1962) Eine eventuelle Stoffwechselschuld ist jedenfalls bei kurzen subdiastolischen Okklusionen nicht grösser, als dass sie auch noch ohne stärkere Mehrdurchblutung gedeckt werden kann denn nach Okklusionen bis zu 20 sec Dauer nahm der Flächenwert der reaktiven Hyperämie — ein relatives Mass für das über den Ruhewert hinaus durchgeflossene Blutvolumen — nur unbedeutend zu (Tab II Fig 7) Erst nach längeren Okklusionen wurde der Flächenwert grösser und stieg in linearer Abhängigkeit zur Okklusionsdauer an *weshalb die reaktive Hyperämie hier vorwiegend auf den zeit* abhängigen Prozess des Stoffwechsels (Muskel) bezogen werden kann

Der sogenannte after-drop kan die reaktive Hyperämie nach subdiastolischer Okklusion nicht erklären Zwar kann dabei der arterio-venöse Druckgradient während 5—20 sec zunehmen und der arterielle Einstrom dadurch bis auf das Doppelte ansteigen (Allwood 1956) Grundsätzlich kann eine solche Erklärung der reaktiven Hyperämie aber schon deshalb nicht überzeugen weil der after-drop" im Gegensatz zur reaktiven Hyperämie mit zunehmender Okklusionsdauer kürzer und kleiner wird

Am Katzenbein verminderten subdiastolische Okklusionsdrucke den arteriellen Einstrom erheblich stärker als am Arm Dennoch kam es dort auch nach 120 sec langen subdiastolischen Okklusionen zu keiner deutlichen reaktiven Hyperämie Gleiches hatten Pritchard et al (1942) und Hilton (1953) bei ähnlichen Messungen an Katzen und Hunden gefunden Der Grund dazu ist unbekannt doch können die Versuchsbedingungen mit Narkose und direkter Messung des arteriellen Einstromes die zu Tonusverminderung der Widerstandsgefässe und Durchblutungserhöhung führen die Reagibilität der Widerstandsgefässe verändert haben (cf Folkow 1952)

Vergleichbarkeit plethysmografischer mit anderen Messungen von arteriellem Einstrom und Druck

Die initiale Verminderung des arteriellen Einstromes war plethysmografisch nicht zu erkennen. Vor jeder anderen Erklärung der Diskrepanz ist daher zu prüfen, ob diese mit anderen Methoden gefundenen Änderungen des arteriellen Einstromes überhaupt mit den gleichzeitig plethysmografisch registrierten Durchblutungswerten vergleichbar waren.

1) Direkte Messungen des arteriellen Einstromes. Gute quantitative Übereinstimmung zwischen direkt und plethysmografisch gemessenen Werten des arteriellen Einstromes ist verschiedentlich gefunden worden: die plethysmografischen Werte waren im Durchschnitt um nur 4 % geringer als die direkt gemessenen (Imig et al. 1955, Formel und Doyle 1957). Solche quantitative Übereinstimmung war in den Versuchen am Katzenbein infolge des Grössenunterschiedes der beiden Messbereiche (Fig. 1) nicht zu erwarten. Die mit beiden Verfahren gefundenen relativen Änderungen des arteriellen Einstromes waren hingegen recht ähnlich (Tab. I). Dieses schliesst zwar nicht die Erklärung der Diskrepanz zwischen eindeutiger Abnahme des arteriellen Einstromes und dennoch gleichförmigem Anstieg der plethysmografischen Einflusskurve dadurch aus, dass sich der arterielle Einstrom im plethysmografischen Messbereich im Unterschenkel während der Registrierung der Einflusskurve anders verhalten haben konnte als im ca. 35 % grösseren direkten Messbereich, doch durfte eine solche Reaktion wenig wahrscheinlich sein.

2) Messung mit Wärmetransport-Zahlmessern.

Muskel. Bei gefässnahen (arteriennahen) Sondenlagen erfasst die Wärmeleitsonde die Durchblutung (arterieller Einstrom) eines Bezirkes, der um das Vielfache grösser sein kann als der ca. 1 ml grosse thermische Messbereich selbst. Daraus erklärt sich die oft gute semiquantitative Übereinstimmung mit anderen (direkten und plethysmografischen) Durchblutungsmessungen (Literatur bei Golenhofen et al. 1963).

Arteriennahe Sondenlagen. Golenhofen et al. (1963) hatten den Durchblutungsabfall der mit der Wärmeleitsonde bei subdiastolischer Okklusion (Venenstauung) zu registrieren ist, nicht für eine arterielle, sondern für eine Reaktion in venösen Gefässen. Neben den üblichen Differenzierungskriterien (siehe „Methodik") wurden aber auch Unterscheidungsmerkmale beobachtet, die gerade bei venöser Stauung oder arterieller Drosselung hervortraten und eine Differenzierung zwischen Durchblutungsreaktionen in vorwiegend arteriellen oder vorwiegend venösen Gefässen der Muskulatur wahrscheinlich machten. *1)* bei arteriennaher Lage betrug bei subdiastolischen Okklusionen am Oberarm der initiale Abfall der Wärmetransportzahl in den ersten 10 sec nicht mehr als 10 % (Tab. II); bei venennaher Lage war er dagegen steiler und grösser (20–50 %) und mehr wahrscheinlich eine Anzeige des plötzlichen venösen Durchblutungsstopps; *2)* bei arteriennaher Lage fiel die Wärmetransportzahl bei Erhöhung des Okklusionsdruckes kontinuierlich weiter bis schliesslich auf den Durchblutungs-Nullwert ab (Fig. 3); bei venennaher Lage hingegen traten bei kompletter Drosselung des arteriellen Einstromes oft wellenförmige Schwankungen auf (wahrscheinlich venomotorisch bedingt); *3)* bei arteriennaher Lage nahm das Ausmass (Flächenwert) der reaktiven Hyperämie nach subdiastolischen Okklusionen von mehr als 20 sec Dauer in linearer Abhängigkeit von der Okklusionsdauer zu (Tab. II, Fig. 7); bei venennaher Lage waren auch nach 2 minutiger Okklusion die reaktiven Durchblutungsanstiege geringer und oft nach 15 sec wieder abgeklungen (und zeigten hier wohl hauptsächlich den Abfluss des venös aufgestauten Blutes an).

Haut. In der Haut ist aus methodischen Gründen eine bevorzugte Erfassung des arteriellen Einstromes mit Wärmetransportzahlmessern kaum zu erwarten (cf. Golenhofen

et al. 1963) Der bei subdiastolischen Okklusionen registrierte Abfall der Wärmetransportzahl in der Haut konnte daher auf einer Verminderung des arteriellen Einstromes ebenso wie auf dem plötzlichen Stopp des venösen Rückflusses oder auf beiden diesen Reaktionen zugleich beruhen

3) Arterieller Einstrom und Druck Subdiastolische Okklusionen am Oberarm bewirkten *noch keine messbaren Veränderungen des distalen arteriellen Druckes was auch andere* Autoren beobachtet hatten (Wilkins und Bradley 1946 Edholm et al 1954 Formel und Doyle 1957) Aus dieser Konstanz des arteriellen Druckes wurde auf einen ebenso unverändert gebliebenen arteriellen Einstrom geschlossen (Greenfield 1957 und 1960 Greenfield et al 1963) Eine solche Schlussfolgerung erscheint aber nicht immer berechtigt. In oszillierenden elastischen Systemen wie den Gefässen können geringe Änderungen des Stromzeitvolumens nur zu minimalen nicht sicher erkennbaren (messbaren) Veränderungen eines einzelnen Druckwertes führen Am Unterarm jedenfalls haben plötzliche Abnahmen des arteriellen Einstromes um 5—10 % noch keine gleichzeitig sicher messbaren Änderungen des lokalen arteriellen Druckes zur Folge

Bedeutung der arteriellen Einstromveränderungen für okklusionsplethysmografische Durchblutungsmessungen

Für die Bewertung okklusionsplethysmografischer Durchblutungsmessungen ist besonders die initiale Abnahme des arteriellen Einstromes von Bedeutung Die sekundären Änderungen des arteriellen Einstromes die erst bei zunehmender Gefässfüllung auftreten haben wahrscheinlich noch keinen direkten Einfluss auf plethysmografische Durchblutungsregistrierungen

Das scheinbare Missverhältnis zwischen tatsächlicher initialer Verminderung des arteriellen Einstromes und dennoch oft linearem Anstieg der plethysmografischen Einflusskurve ist nicht einfach zu erklären Die Diskrepanz kann nicht damit begründet werden dass die plethysmografischen Einflusskurven für die Anzeige der in Tab I und II beschriebenen arteriellen Einstromsverminderungen ungeeignet zu unempfindlich waren So hatte beispielsweise am Arm (bei einer mittleren Durchblutungsgrösse von 3 ml 100 ml min) die in den ersten 10 sec registrierte Einstromverminderung von 5 % den Anstieg der Einflusskurve in dieser Zeit um ca 0 025 Vol % (ml 100 ml) gegenüber dem linearen Verlauf vermindern müssen abszissenkonkaver Kurvenanstieg was durchaus noch messbar sein kann Graf 1964 b) Mit Sicherheit aber wären die grösseren Einstromverminderungen am Katzenbein von 20—30 % plethysmografisch erfasst worden wo bei einer mittleren Durchblutung von 15 ml 100 ml min) der Anstieg der Einflusskurven in den ersten 10 sec um ca 0 50—0 75 Vol % (ml 100 ml) gegenüber dem linearen Verlauf vermindert worden wäre aber auch hier stiegen die Einflusskurven in dieser Zeit meist linear an (Fig 2) Eine weitere Erklärungsmöglichkeit läge darin dass sich am Katzenbein der arterielle Einstrom in den plethysmografischen Messbereich während der Registrierung der Einflusskurve anders verhalten haben könnte als im ca 35 % grösseren direkten Messbereich die weitgehende Ähnlichkeit der mit beiden Methoden registrierten relativen Einstromänderungen macht aber auch diese Annahme unsicher

Am wahrscheinlichsten bleibt somit eine Reaktion, die auch schon als die unwahrscheinlichste bezeichnet worden ist („it is difficult to imagine a mechanism which could instantaneously change the peripheral resistance to a new and steady level. An instantaneous change in the rate of inflow is therefore most unlikely". Greenfield 1957), dass nämlich die arterielle Einstromverminderung initial sehr plötzlich erfolgte und somit von der Einflusskurve noch gar nicht angezeigt werden konnte. Die Registrierungen des arteriellen Einstromes mit Wärmeleitmessern und Tropfenzähler hatten in diesen Untersuchungen eine durchschnittliche Trägheit bzw. Anzeigeverzögerung von mehreren (2—3) sec, weshalb der sich über mehrere Sekunden hinziehende initial steile Abfall des arteriellen Einstromes in Wirklichkeit rascher erfolgte. Diese Annahme wird auch durch die Befunde von Conrad und Green (1961, Fig. 3) bestärkt, die bei subdiastolischer Okklusion am Hundebein mit einem electromagnetic flowmeter einen initialen, in weniger als 0,5 sec beendeten Abfall des arteriellen Einstromes um 7 % registriert hatten. Die plötzliche initiale Verminderung des arteriellen Einstromes ist somit am ehesten mit einer partiellen arteriellen Okklusion (*Verengerung*) *durch den subdiastolischen Okklusionsdruck zu erklären*. Eine Erklärung der plötzlichen Widerstandserhöhung durch eine Zunahme des Venendruckes ist kaum möglich, weil dieser nicht so rasch ansteigen konnte: die Versuchsbedingungen mit ausreichender Hochlagerung des Unterarmes sowie die zumindestens in den ersten 5 sec nach Okklusionsbeginn stets linear angestiegenen plethysmografischen Einflusskurven schliessen eine von Beginn an stärkere Füllung der kapazitiven (venösen) Gefässe und damit die Möglichkeit eines raschen initialen Anstieges des Venendruckes praktisch aus (cf. Greenfield und Patterson 1954). Ein allmählicher Venendruckanstieg kann aber zu der während der ersten 10 sec der subdiastolischen Okklusion successiv erfolgten geringeren Verminderung des arteriellen Einstromes beigetragen haben.

Die reaktive Hyperämie nach subdiastolischen Okklusionen bestimmt den Zeitabstand aufeinanderfolgender plethysmografischer Durchblutungsmessungen. Da nach den üblichen venösen Okklusionen am Oberarm die reaktive Hyperämie im Unterarm nicht länger als 5—10 sec anhält, ist dies die Zeit, die dort bis zum Beginn der nächsten Messung abgewartet werden muss. Diese Wartezeit stimmt mit der üblichen plethysmografischen Registrierpraxis überein (Graf 1964 b).

Die schon durch subdiastolische Okklusionsdrucke bewirkte partielle Okklusion der arteriellen Gefässe ist ein typischer „reactive error of the method" im Sinne Burtons (1954). Am Arm ist dieser Fehler unter normalen und Ruhebedingungen (Tab. II) jedoch noch relativ gering und stört die Registrierungen selbst nicht, weshalb er wohl in den meisten Fällen toleriert werden kann. Bei verminderter Elastizität der Arterien (etwa bei Arteriosklerose: Mackay und Walker 1963) ist die initiale Einstromverminderung wahrscheinlich geringer, während sie bei höherer Durchblutung oder bei anderen mit erhöhter Elastizi-

tat oder elastischer Dehnung der Arterien einhergehenden Zuständen vermutlich grosser werden kann. Trotz dieses reactive error kann aber die venöse Okklusionsplethysmografie weiterhin als die vor allem am Menschen und dort besonders zu Vergleichsmessungen praktisch meist anwendbare und genaueste Methode zur unblutigen, quantitativen Bestimmung des arteriellen Einstromes in Extremitätenabschnitten angesehen werden.

Die Untersuchungen erfolgten mit Unterstützung durch den Schwedischen Medizinischen Forschungsrat und die Schwedische Nationalvereinigung zur Bekämpfung von Herz- und Lungenkrankheiten. Der AB Elema, Stockholm (Direktor G. Weber und Dr. R. Elmqvist) sei für die freundliche Bereitstellung apparativer Ausrüstung, Frau I. Graf für Assistenz bei der Auswertung der Versuche gedankt.

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The Accumulation and Localization of Radioactive ^{60}Cobalt in Rat Kidney

By

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Abstract

KASANEN, A., I. LINDGREN and H. A. SALMI. *The accumulation and localization of radioactive cobalt in rat kidney.* Acta physiol. scand. 1964. 61. 376—379. — The accumulation of radioactive cobalt after intramuscular injection was followed in rats, and its distribution in kidney was studied using autoradiographs. The radioactivity accumulated rapidly in kidney tissue and its localization was the renal cortex in the vicinity of the cortico-medullary zone forming wedge formed areas against the renal capsule. Most of the activity was in the tubuli. No activity was observed in the medulla or the glomeruli.

It has been shown that oral and intramuscular cobalt therapy has a beneficial effect on renal anemia (Gardner 1953, Kasanen *et al.* 1962 and Kasanen *et al.* 1963). However, the effect is seen only in a part of renoprive anemias. It has been assumed that the effect of cobalt is the inhibition of the oxydative enzymes in the bone marrow inducing a secondary polycythemia (Orten *et al.* 1948). On the other hand, it has been suggested that cobalt stimulates humoral erythropoetic factor (Jacobson *et al.* 1960). A reduced level of humoral erythropoietic factor has been found in patients suffering from renal anemia (Goldwasser *et al.* 1958 and Jacobson *et al.* 1960). The kidney has been said to produce an inactive precursor which is activated by inorganic cobalt (Jacobson 1960).

The following is a report of a study on the accumulation and localization of radioactive cobalt in healthy rat kidney.

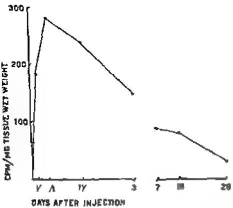

Fig 1 The accumulation of rad oactivity in rat k dney tissue after intr amuscular inject on of ^{60}CoCl

Methods

Female Wistar rats served as test animals The body weights of the 7 rats at the be ginning of the experiment were 260—300 g ^{60}CoCl (cobaltous chloride marked with ^{60}Co specific activity 1 mc/20 μg of cobalt The Radiochemical Centre Amersham England) A dose of 100 μc dissolved in 0 5 ml of saline was injected into each animal The rats were killed 4 and 12 hours and 1 1/2 3 7 14 and 24 days after the single in jection

The kidneys were dissected and weighed One kidney was fixed in 10 per cent formalin and prepared according to conventional methods and embedded in paraffin A slice through the cortex into the pelvis was cut out from the other kidney This slice was weighed and treated with sulphuric acid The radioactivity of this solution was measured with a welltype scintillation counter using a voltage of 690 and discrimination bias of 35

Sections 5—7 micra thick were taken from paraffin blocks The sections were mounted on clean glass slides and the autoradiographic stripping film technique was used Two types of photographic material were employed Kodak AR 50 for low re olution auto radiography and Kodak AR 10 for high resolution autoradiography More than 160 slides were prepared The exposure time varied from 1 to 3 months The films were developed in Kodak D 170 solution (20 C 3 min) and fixed in the ordinary photographic way The slides were stained with hematoxylin and celestin blue

Results

The cumulation of radioactivity in renal tissue and its disappearance there from is illustrated in Fig 1 The rapid rise and the slow decrease of the radio activity are conspicuous

The localization of the radioactivity can be seen in Fig 2 and 3 Fig 2 presents a magnification of an autoradiographic picture in which the renal cortex and medulla are seen in the same section The greatest activity (the blackest areas in the picture) is concentrated most densely near the cortico medullary zone From here the dark areas (radioactivity) form wedge shaped zones approaching the renal capsule There is no activity in the medulla nor

Fig. 2. Rat kidney one week after ^{60}CoCl injection. The big arrow (A) in the medulla of one papilla indicates the medulla of another lobe. No silver grains at all are seen in the medulla of the kidney. The black areas seen above this are caused by silver grains which show activity only in the cortex. This activity is most markedly localized in the cortico-medullary boundary from where it as irregular pyramid like areas is diminishing towards the cortex. The small arrows indicate blood vessels with no activity. Correspondingly no activity is established in the medullary striae. Low resolution autoradiogram magnification 15 ×

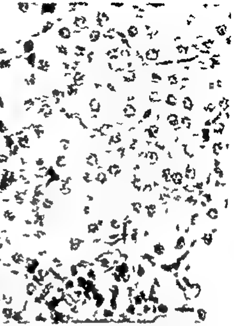

Fig. 3. The rat kidney one week after ^{60}CoCl injection. The arrows indicate a glomerulus with no activity. Only little activity is seen in the collecting tubuli. The greatest activity lies in the tubuli. High resolution autoradiogram. Magnification 650 ×

in the blood vessels near the boundary of the cortex and medulla. This localization was noted in all the low resolution autoradiograms. With high resolution autoradiographs it could be shown that the most activity was localized in the tubuli nearest to the cortico-medullary boundary. All the tubuli showed some activity. The weakest activity was seen in the tubuli nearest the renal capsule. No activity was seen in the glomeruli.

Comment

Using scintillation counting and macroscopic autoradiographs Carlberger (1961) showed that the kidney was one of the main organs for the accumulation of radioactive cobalt. The present observations demonstrate that cobalt localizes in the tubuli and especially in the tubuli near the cortico-medullary

boundary and gradually decreases from here towards the renal capsule The localization is inverse to that of the radioactive cyanocobalamin both in foetal and in adult rat kidney (Salmi 1963 Salmi and Lindgren 1963)

The distribution of these two hematopoietically active substances, cobalt and cyanocobalamin differs Both these substances play a role in hematopoiesis and the cyanocobalamin molecule also incorporates a central cobalt atom However these substances are deposited differently in the renal cortex Because the autoradiographic technique used in present work discloses water soluble cobalt the present findings suggest that cobalt possibly has a role in tubular metabolism

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Fig 2 Rat kidney one week after $^{60}CoCl$ injection The big arrow (↘) in the medulla of one papilla indicates the medulla of another lobe No silver grains at all are seen in the medulla of the kidney The black areas seen above this are caused by silver grains which show activity only in the cortex. This activity is most markedly localized in the cortico-medullary boundary from where it as irregular pyramid like areas is diminishing towards the cortex. The small arrows indicate blood vessels with no activity Correspondingly no activity is established in the medullary striae Low resolution autoradiogram magnification 15 ×

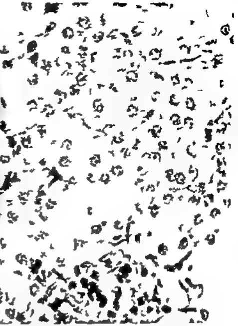

Fig 3 The rat kidney one week after $^{60}CoCl$ injection The arrows indicate a glomerulus with no activity Only little activity is seen in the collecting tubuli The greatest activity lies in the tubuli High resolution autoradiogram. Magnification 650 ×

in the blood vessels near the boundary of the cortex and medulla This localization was noted in all the low resolution autoradiograms With high resolution autoradiography it could be shown that the most activity was localized in the tubuli nearest to the cortico-medullary boundary All the tubuli showed some activity The weakest activity was seen in the tubuli nearest the renal capsule No activity was seen in the glomeruli

Comment

Using scintillation counting and macroscopic autoradiography Carlberger (1961) showed that the kidney was one of the main organs for the accumulation of radioactive cobalt The present observations demonstrate that cobalt localizes in the tubuli and especially in the tubuli near the cortico-medullary

radioactively labelled nicotine and thin layer chromatography to identify some of the breakdown products and measure their quantitative significance The mouse was chosen as the experimental animal since our previous distribution studies were performed in this species

Methods

Nicotine methyl C^{14} was synthesized according to the method by McKennis et al 1962 as described by Hansson and Schmiterlow (1962) and had a specific activity of 61 μC/mg Radiochemical purity was established in the first two of the solvent systems described below Samples of cotinine hydroxycotinine γ (3-pyridyl) γ-oxo-N methyl butyramide and desmethylcotinine were kindly donated by Dr Herbert McKennis Jr Richmond Va A sample of 3 (2 (1 methylpyrrolino) pyridine 1-oxide (nicotine N oxide) was generously supplied by Mr E Haglid Kungl Tekniska Hogskolan Stockholm. All other reagents were of reagent grade

Male mice weighing 20 – 25 g were used Tissue slices of approximately 0 3 mm thickness were prepared free hand with a razor blade The incubation medium was Krebs Henseleit phosphate buffer pH 7 4 containing glucose at a concentration of 0 01 M All experiments were performed at 37 C in a Warburg apparatus For aerobic conditions the gas phase was 100 % O_2 while anaerobiosis was achieved by gassing with 100 % N_2 Each flask contained 2 0 ml of medium while the center well contained a filter paper strip soaked in 5 N NaOH for collection of $C^{14}O_2$ The side arm contained 0 5 ml 0 2 N HCl which was tipped into the main compartment at the end of the experiment to liberate retained CO_2 Between 100 and 200 mg (wet weight) of tissue were usually added to each flask ( ) Nicotine methyl C^{14} was added to the tissue slices and after gassing for 5 min the incubation was continued for 2 hours in most cases At the end of the incubation the acid in the side arm was tipped into the main compartment and the flasks allowed to shake for an additional 10 min in order to collect residual $C^{14}O_2$

The contents of the flask including the tissue slices were transferred to a 15 ml Potter Elvehjem homogenizer The flasks were rinsed with 1 0 ml water and 0 5 ml 0 5 N ammonium hydroxide (bringing pH to 9) which was added and the tissue homogenized The homogenate was extracted in a 60 ml separatory funnel with 10 ml of chloroform methanol (2 1) (v/v) (Papadopoulus and Kintzios 1963) and the two phases separated by centrifugation at 1 500 × g Radioactivity was determined on 0 1 ml aliquots of each phase by liquid scintillation counting after addition of 15 ml of 0 5 2 5-diphenyl oxazole (DPO) + 0 03 1 4 bis-2(4-methyl 5-phenyloxazolyl) benzene (dimethyl POPOP) in toluene

The solvent system for thin layer chromatography found to give the most complete separation of metabolites present in the chloroform phase of the tissue extracts was ethanol acetone-benzene-conc NH_4OH (5 40 50 5) (v/v) This system separated nicotine cotinin (3 pyridyl) γ-oxo-N methylbutyramide nicotine N-oxide and hydroxycotinine Desmethylcotinine ran with γ (3 pyridyl -γ-oxo-N methylbutyramide in this system but this presented no problem in the present study since the C^{14} label is in the methyl group and thus desmethylcotinine does not produce a spot on the autoradiogram A second solvent system was used to check the results, namely chloroform-ethanol (90 10) (v v) The plates used with this system were prepared with 0 5 N NaOH instead of water This system gave good separation of the above compounds with the exception of nicotine and cotinine which ran together Chromatographic investigation of the methanol water phase of the tissue extracts was performed on thin layer plates developed with water as the solvent system and with phenol water (80 30) (w/v In

all cases the front was allowed to run for approximately 14 cm Development time for the chromatograms was between 20 and 30 min with the exception of the last system which required about $2^1/_2$ hours The solvent front ran straighter and the development times were shorter when the walls of the chromatography chamber were covered with filter paper soaked in the solvent Non radioactive standard compounds were located by spraying the plates with p aminobenzoic acid in 96 % ethanol (1 % w/v) and exposing the plates to cyanogen bromide vapor Nicotine N oxide was located by exposure to iodine vapor

An aliquot of the chloroform phase usually 0 5 to 1 0 ml was reduced in volume under N_2 and spotted on 250 μ thick silica gel thinlayer plates for chromatography The plates were prepared as described by Stahl (1962) and were activated by heating at 110 C for 30 min

After drying of the plate by allowing it to stand in the air radioactive compounds were located by exposing the plate to Kodak no screen X ray film for 2 and 8 days

Viewing by means of an ordinary X ray film viewer of the autoradiograms upon which the thin layer plates were superimposed allowed the radioactive spots to be marked These areas where then scraped off with a razor blade into 20 ml liquid scintillation vials and counted as a suspension in 0 5 % 2 5 DPO + 0 03 % dimethyl POPOP + 4 % silica gel (Aerosil) in toluene (Snyder and Stephens 1962) Corrections for quenching were made by using n hexadecane 1 C^{14} dissolved in toluene as the internal standard

The NaOH impregnated filter paper was removed from the center well put in a liquid scintillation vial and dried overnight over silica gel Fifteen ml of 0 5 % 2 5 DPO + 0 03 % dimethyl POPOP in toluene were added (Buhler 1962) and the sample counted in a liquid scintillation counter

Results

Recoveries of added radioactivity were between 90 and 95 % for controls run through the procedure without tissue and for those tissues showing little or no capacity for metabolizing nicotine In case of liver slices the combined mean recoveries in the chloroform methanol water and $C^{14}O_2$ fractions ran between 80 and 85 %

Approximately 97 % of the activity in the chloroform aliquot applied to the thin layer plate could be recovered in the various radioactive spots found after chromatographic development There was a linear relationship between the total amount of the chloroform extract spotted on the chromatogram and the activity recovered in the various radioactive spots The sensitivity of the method is indicated by the fact that the presence of approximately 500 dpm could be detected on the autoradiogram after an exposure time of 2 days With the specific activity of 61 μC/mg nicotine used in these experiments this corresponds to 3 7 nanograms or less than 0 1 % of the activity added to the slices Despite rigorous attempts to standardize the preparation of the thinlayer plates and the development conditions and despite the use of fresh solvents with each run it was not possible to obtain reproducible R_f values from plate to plate The relative position of the various metabolites upon the chromatogram was however, constant, as was their position in relation to that of nicotine the substance

Fig 1 Autoradiogram of thin layer chromatogram of chloroform phase of extract of mouse tissue slices Solvent system ethanol acetone benzene conc NH_4OH (5-40 50 5) (v/v) Solvent front = 14 cm Exposure time = 2 days Components nicotine (R_f = 0 71) autooxidation product (0 55) cotinine (0 47) γ (3 pyridyl) γ-oxo-N methylbutyramide (0 32) X (0 29) hydroxycotinine (0 22) Y (0 11)

with the highest R_f (compare Fig 1 and Fig 3) Standard C^{14} nicotine and non radioactive reference substances were always run on each plate so that identification of the compound on the plate was always the results of comparison with these standards and positive results with co chromatography

Incubation of nicotine methyl C^{14} under the prescribed conditions under 100 % O_2 in the absence of tissue resulted in the appearance of minute quantities of autooxidation products of nicotine on the autoradiogram That these spots were the results of autooxidation was indicated by the fact that nicotine methyl C^{14} applied directly to the plates (designated standard nicotine) and chromatographed always showed only one radioactive spot on the autoradiogram On some autoradiograms as many as 10 radioactive spots could be detected in the chloroform fraction from the control following incubation for 2 hours at 37 C The amounts of these products formed varied from 0 05 % to as much as 0 2 % of the total activity in the most extreme case

Of those mouse tissues tested the liver the kidney and the lung showed an appreciable ability to metabolize nicotine in a 2 hour incubation period The brain spleen diaphragm intestine stomach and adrenal showed no or only insignificant capacity to metabolize the added compound in 2 hours (Table I and II)

In the chloroform fraction from the liver slices 6 radioactive spots could be seen on the autoradiogram of the thin layer chromatogram after incubation for

Table I Distribution of radioactivity from ni otine C^{14} into various chemical fractions following incubation with tissue slices Slices incubated for 2 hours at 37 C in Krebs Henseleit buffer under 100 O 5 1 µg nicotine methyl C^{14} equivalent to 600 000 dpm in each flask

Tissue	Wet weight (mg)	Percentage of total radioactivity recovered		
		Chloroform	Methanol water	C O
Control (no tissue)	—	97 7	2 3	—
Liver	349	78 9	16 7	4 4
Kidney	224	87 5	4 8	7 7
Lung	111	84 7	15 1	0 15
Spleen	218	97 6	2 4	—
Diaphragm	106	95 8	3 8	0 4
Intestine	179	97 0	2 9	0 1
Stomach	130	97 8	2 1	—
Adrenal	12	97 3	2 6	—
Brain	230	97 8	2 2	—

Table II Distribution of radioactivity between metabolites found in the chloroform phase of the extract of tissue slices from various mouse organs Slices incubated for 2 hours at 37 C in Krebs Henseleit buffer under 100 O 5 1 µg nicotine methyl C^{14} equivalent to 600 000 dpm in each flask

	Nicotine	Cotinine	γ (3 pyridyl) γ oxo N methyl butyramid	X	Hydroxy cotinine	X	Origin
Control (no tissue)	97 6	—	—	—	—	—	2 0
Liver	9 9	73 3	2 4	4 0	9 0	1 8	—
Kidney	88 3	3 0	0 8	—	—	1 0	2 8
Lung	97 8	1 0	—	—	—	0 4	0 [illegible]
Spleen	98 1	—	—	—	—	—	0 [illegible]
Diaphragm	97 5	—	—	—	—	0 4	0 [illegible]
Intestine	9[illegible] [illegible]	—	—	—	—	0 2	0 4
Stomach	[illegible] 0	—	—	—	—	0 2	0 6
Adrenal	97 0	—	—	—	—	0 [illegible]	1 [illegible]
Brain	9[illegible] 6	—	—	—	—	0 [illegible]	0 9

2 hours (Fig 1) Four of
raphy with standard
is by far the most impo
of the total activity
hydroxycotinine a

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about 75 %
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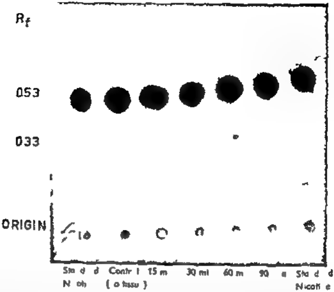

Fig 2 Autoradiogram of thin layer chromatogram of chloroform phase of extract of mouse liver slices under anaerobic conditions Tissue weight = 200 mg Solvent system ethanol acetone benzene-conc NH OH (5-40 50 5) (v/v) Solvent front = 14 cm Exposure time = 2 days Components nicotine (R_f = 0 53) cotinine (0.33)

1) although appearing regularly in the 12 experiment performed with liver slices were present in small amounts accounting together for 13 % of the activity present in the chloroform fraction of the liver slices The amount of $C^{14}O$ found was equivalent to about 1 5 % of the added activity

It can be noted that the autooxidation product with an R_f of 0 53 present in the control and seen also in the other tissues is not present in the extract from the liver (Fig 1) It would appear that this product is subject to further degradation in the liver

Chromatography in the chloroform ethanol system showed the presence of the same metabolites as the routinely used ethanol acetone benzene-conc ammonium hydroxide system The amounts of radioactivity in the various metabolites separated by each of the two systems were identical within 2

Further attempts to identify the compound here called "X (R_f = 0 29) and "Y (R_f = 0 11) have up to the present been unsuccessful Nicotine N oxide has been ruled out as one of these metabolites on the basis of its failure to co-chromatograph with the radioactivity The hydrolysis product of cotinine γ (3 pyridyl) γ methylaminobutyric acid remains at the origin in this solvent system and may account for some of the activity seen at the origin although on the basis of its acidic properties one would expect to find it in the methanol water phase.

Table III Quantitative data from the chromatogram illustrated in Fig 2 and respiratory $C^{14}O$ showing the oxygen dependence of nicotine metabolism in mouse liver slices Slices incubated for the indicated time periods at 37 C in Krebs Henseleit buffer under 100 \ 5 1 μg nicotine methyl-C^{14} equivalent to 600 000 dpm in each flask

Incubation time	Per cent of total radioactivity in chloroform phase Control 90 min	15 min	30 min	60 min	90 min
Nicotine (0.53)	97 3	98 5	93 5	98 4	98 6
Cotinine (0.33)	0.2	0 4	0 5	0 6	0 5
Origin	1.9	0 4	0 3	0 4	0.3
$C^{14}O$ (total dpm)	0	33	0	[illegible]	45

Table II Quantitative data from the chromatogram illustrated in Fig 3 as well as $C^{14}O$ showing the time course of nicotine metabolism in mouse liver slices Data for the kidney taken from chromatogram not shown Slices incubated for the indicated time periods at 37 C in Krebs Henseleit buffer under 100 O 5 1 μg nicotine methyl C^{14} equivalent to 600 000 dpm in each flask

Incubation time	Percentage of total radioactivity in chloroform phase Control 90 min	15 min	30 min	60 min	90 min
Liver					
Nicotine	97 6	85 9	80 0	64 8	55 0
Cotinine	1 1	11 5	16 7	30 4	38 6
-(3 pyridyl)-oxo-N methylbutyramide	—	0 4	0 6	1 3	2 0
"X"	—	0.25	0 3	0 7	1 0
Hydroxycotinine	—	0 3	0 4	0 7	0 9
"Y"	—	0 3	0 7	0 7	1.2
$C^{14}O$	—	0 9	0 9	1 2	1.8
Kidney					
Nicotine	97 7	96 7	95 5	94 5	93.8
Cotinine	—	0 4	1 0	2.3	3.2
-(3 pyridyl)-)-oxo-N-methylbutyramide	—		0 1	0.2	0.3
$C^{14}O$	—	1 1	1 4	1 6	2

Because of the selective position of the C^{14} label on the methyl group of the pyrrolidine ring nitrogen the presence of demethylated products of nicotine metabolism that have been found in the urine of various species (desmethyl cotinine , (3 pyridyl) ,-oxo-butyric acid and 3-pyridylacetic acid McKennis et al 1961 1962 and Schwartz and McKennis 1963) would not have been detected by us on the autoradiograms of the thin layer chromatograms The

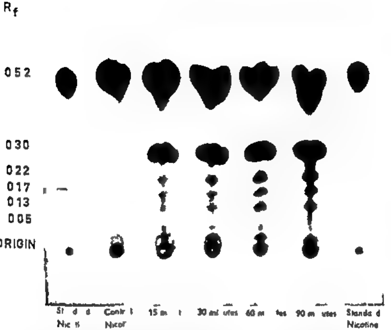

Fig 3 Autoradiogram of thin layer chromatogram of chloroform phase of extract of mouse liver slices under aerobic conditions showing metabolism as a function of time Tissue weights = 200 mg Solvent system ethanol acetone benzene-conc NH_4OH (5-40 50-5) (v/v) Solvent front = 14 cm Exposure time = 2 days Components nicotine (R_f = 0.52) cotinine (0.30) γ (3 pyridyl) γ-oxo-N methylbutyramide (0.22) X (0.17) hydroxycotinine (0.13) Y (0.05)

amount of $C^{14}O_2$ can however be taken as an indication of the extent of the reactions involving demethylation

When the liver slices were incubated under anaerobic conditions (100 % N_2) *for varying lengths of time (15—90 min) there was almost quantitative recovery* of unchanged nicotine from the incubation mixture (Fig 2) There were very small quantities of cotinine formed 0.3 % in 90 min compared with 38.6 % in 90 min under 100 % O_2 (Table III) The control without tissue also showed virtually only one spot in contrast to the aerobic controls lending support to the notion that the minor spots in the latter were due to autooxidation of the nicotine

The incubation of liver slices for time periods varying from 15 to 90 min resulted in a steady fall in the amount of unchanged nicotine with a concomitant *increase in the concentrations of cotinine and the other metabolites present in* the chloroform fraction (Table IV Fig 3) It can be noticed that the quanti

Table I Effect of iproniazid on nicotine metabolism in mouse liver slices incubation time = 2 hours

	Wet weight (mg)	Nicotine	Cotinine	γ (3 pyridyl) γ oxo-N methyl butyramide	X	Hydroxy cotinine	Y	Origin
Control (no tissue)	—	96 6	1 2	—	—	—	—	2 2
Liver slices	200	28 9	60 4	3 1	2 3	2 1	1 6	1 6
Liver slices + 4×10^{-4} M iproniazid	207	22 5	62 5	5 1	3 5	2 8	1 6	2 0
Liver slices + 4×10^{-3} M iproniazid	208	89 3	5 7	1 1	0 4	0 5	0 4	2 5

tative significance of the metabolites other than cotinine is minor amounting to 5 % of the total activity though their concentration increases with time throughout the time period studied (Table IV)

A similar picture is seen in case of the kidney although hydroxycotinine and one of the unidentified metabolites (X) do not appear to be formed in this system (Table II Fig 1 Table IV) The kidney was found to produce amounts of cotinine and γ (3 pyridyl) γ oxo N methylbutyramide along with $C^{14}O_2$ The capacity of the kidney slices to metabolize nicotine was not as great on a weight basis as that of the liver However the amount of $C^{14}O_2$ produced by the kidney was almost 3 times that of the liver on a per mg wet weight basis (Table I)

The other tissue found to metabolize nicotine to a significant extent was the lung Here as in the other tissues the main metabolite was cotinine no other metabolites appearing in detectable concentrations (Table II)

As shown in Table II the other tissues tested showed no appreciable metabolism of nicotine as indicated by the presence of only one spot corresponding to nicotine on the autoradiograms of the chloroform fraction and the practically 100 % recovery in the chloroform phase when compared with the control (Table I) The diaphragm did produce some $C^{14}O_2$ amounting to 0 3 % of the added activity over a 2 hour period This was about 1/10 of the activity found in the liver and about 1/30 of that found in the kidney No amounts of cotinine or other labelled products found in the liver could be detected in the chloroform extracts of the diaphragm

In experiments designed to detect the presence of radioactively labelled methylamine a possible intermediate in the metabolism of nicotine liver slices were incubated in the same manner under oxygen in the presence of a monoamine oxidase inhibitor iproniazid in an attempt to inhibit the oxidation of any methylamine formed At an iproniazid concentration of 4×10^{-4} M there was no detectable effect on nicotine metabolism The presence of 4×10^{-3} iproniazid inhibited nicotine metabolism by about 85 % (Table V)

An examination of both the chloroform and methanol water phases of the extract from the iproniazid treated liver slices after addition of carrier methylamine failed to detect unequivocably radioactive methylamine In the phenol water system a distinct radioactive spot was seen from the methanol water phase which had an R_f value quite close to that of methylamine However the identification was complicated by tailing and the presence of other ninhydrin reacting components in the extract The possible role of methylamine in nicotine metabolism and the effect of iproniazid on the metabolism of nicotine and methylamine requires further investigation

Investigations of the methanol water phase of the extract up to the present time have been of a limited nature Chromatography of aliquots of this phase from the liver extract in the ethanol acetone benzene conc NH_4OH system showed that over 90 % of the radioactivity remained at the origin and that very little if any of the radioactivity in this phase was due to incomplete extraction of the chloroform soluble metabolites Chromatography on silica gel plates using water as a solvent showed two major diffuse zones of radioactivity with R_f values of 0 04 and 0 56 Similar chromatography using phenol water (80—30) (v/v) as solvent showed four radioactive zones two of which were well localized and two of which showed pronounced tailing Further work will be needed to determine the nature of the radioactivity in this fraction

Discussion

The metabolic products of nicotine noted in this investigation have all been found to be present in the urine of animals of various species to which nicotine had been administered (McKennis 1960) In addition cotinine had been detected earlier on paper chromatograms of chloroform extracts of mouse liver and brain following the intravenous injection of nicotine C^{14} (Hansson and Schmiterlow 1962) The failure of the paper chromatograms to disclose the presence of the other two identified metabolites and the two unidentified ones found in the present investigation can probably be ascribed to the very small amounts of these metabolites that are formed in the liver as well as the increased sensitivity of thin layer chromatography as compared to paper chromatography To this must be added the possibility of the rapid excretion of these compounds into the urine in vivo while the in vitro system being a closed one allows for the accumulation of the metabolites

As mentioned previously because of the position of the label in our nicotine the presence or absence of the demethylated products of nicotine metabolism could not be detected However, the appearance of $C^{14}O_2$ can be taken as a measure of the overall magnitude of these pathways without indicating the exact nature of the concomitantly formed products The presence of desmethyl cotinine γ (3 pyridyl) γ oxo-butyric acid and 3 pyridylacetic acid has been demonstrated in the urine of rats and dogs From the amount of $C^{14}O_2$ produced in the liver kidney and lung in the present investigation these pathways must be considered to be of no major importance at least in vitro McKennis et al 1962 Hansson and Schmiterlow (1962) have shown that in vivo the $C^{14}O_2$ produced amounts to 10—15 % during a 24 hour period in mice and rats It is of interest that while the kidney is much less active in forming cotinine from nicotine than the liver its ability to produce $C^{14}O_2$ is about 3 times that of the liver on a weight basis Whether this difference is the result of a more active formation of nornicotine in the kidney or to differences in the activities of the enzyme systems responsible for the demethylation of cotinine and subsequent oxidation of the methyl fragments to CO_2 is not indicated by our results

The investigation of Hucker et al (1960) has demonstrated that the enzyme activity involved in the oxidation of nicotine to cotinine is in the microsome fraction of the liver and is TPNH dependant In addition Papadopoulus and Kintzios (1963) have shown that the 9 000 × g fraction of liver extracts from the rabbit in addition to forming cotinine are capable of yielding nornicotine and desmethylcotinine from nicotine Four other metabolites were present but unid entified It is the object of further study in this laboratory to determine if the minor metabolites found in the liver slices are formed in the same microsomal fraction as where the main reaction occurs

Our results agree with those of Miller and Larson (1953), who by determining the amount of unchanged nicotine in mouse tissue slices found that the liver lung and kidney were those tissues able to metabolize nicotine It is apparent that the liver is the most active organ with regard to nicotine metabolism

The presence of C^{14} labelled cotinine in the brain of mice injected i v with C^{14} nicotine (Appelgren et al 1962) made it of interest to determine if this transformation could take place in the brain itself since the formation of coti nine in the brain from nicotine might serve as a mechanism for the rapid dis appearance of nicotine from the central nervous system and the termination of its central action On the basis of the in vitro results reported here it appears that the brain itself is not able to form cotinine from nicotine and in fact 97 % of added nicotine can be recovered unchanged after 2 hours of incubation A very small radioactive spot designated Y amounting to 0 5 % of the added activity can be seen on autoradiograms of the chloroform extract from the brain (Fig 2) This spot with a low R_f value has as yet not been identified The same spot has been seen in extracts of the other tissue slices and its quantitative sig

nificance is very minor ranging in amount from 1 8 % in the case of the liver to 0 2 % in the case of the intestine

In earlier unpublished experiments investigating the capacity of the brain to metabolize nicotine rats were eviscerated and hepatectomized and C^{14} nicotine injected intravenously After 15 min the animals were sacrificed and the brains analysed by the above methods for the presence of cotinine in the brain In the hepatectomized eviscerated animals cotinine was indeed found in the brain although in amounts about one half that found in sham operated animals The significance of the results became questionable when it was determined that lung tissue slices were capable of forming cotinine from nicotine and further studies of this nature were discontinued Thus on the basis of the present study and keeping in mind that the results in vitro may not reflect the brain s in vivo capacity to perform this metabolic reaction it would appear that the metabolism of nicotine to cotinine in the brain itself cannot account for the rapid disappearance of nicotine from the brain On the other hand this does not exclude the possibility that cotinine a compound with very little pharmacological activity formed e g in the liver and apparently transported to the brain (Bowman et al 1964) may be involved in a more complex manner with the ultimate distribution of nicotine in the brain

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The Effect of a Ganglionic Blocking Agent on the Thermoregulatory Response to Preoptic Cooling

By

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Abstract

ANDERSSON, B., A. H. BROOK, C. C. GALE and B. HOKFELT. *The effect of a ganglionic blocking agent on the thermoregulatory response to preoptic cooling.* Acta physiol. scand. 1964. 61. 393—399. — A ganglionic blockade obtained by i.v. injection of chlorisondamine 0.2 to 0.3 mg/kg prevented the rise in body temperature which normally occurs during local cooling of the preoptic/anterior hypothalamic region (the "heat loss center") of unanesthetized goats. The shivering response to such cooling was markedly reduced, and instead of the usual rise in urinary excretion of adrenaline and noradrenaline catecholamine excretion remained below normal levels. The thyroid activation obtained by local cooling of the "heat loss center" was not altered by the ganglionic blockade. An i.v. infusion of noradrenaline or adrenaline (0.5 μg/kg/min) counteracted the effects of the ganglionic blockade on the response to central cooling, and further induced marked shivering.

It was recently shown that local cooling of the preoptic/anterior hypothalamic region (the "heat loss center" of Magoun et al. 1938) increased the production of sympathetic amines in non anesthetized goats, whilst warming of the same part of the brain inhibited the activation of the sympathico-adrenomedullary system normally occurring during a general cold stress (Andersson et al. 1964). It was suggested that the "heat loss center" exerts a brake on all neural and hormonal cold defence mechanisms, including the sympathico-adrenomedullary system.

To evaluate further the importance of this system in thermoregulation, it seemed of interest to study the effect of a ganglionic blocking agent on the thermoregulatory responce to local cooling of the "heat loss center", and further, to see whether this effect might be counteracted by the administration of either noradrenaline or adrenaline.

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Methods

Two adult female goats Andrew (b w 45 kg) and Cassius (b w 33 kg) were u ed Silver thermodes for local cooling were permanently implanted medially in the preoptic/anterior hypothalamic region of both animals The method of implantation and the technique of central cooling were previously described (c f Andersson et al 1963) During all experiments the animals remained in their usual environment in metabolism cages, Room temperature was maintained at 18 C (± 1) Ear surface temperature (as index of peripheral blood flow) was measured by a thermocouple attached to the dorsal surface of one ear near its tip To avoid the excitatory effect of application of muscle leads no electromyograms of shivering were taken The presence of even weak shivering however was easily detectable and the intensity of shivering was therefore subjectively graded

All injections and infusions were made via polythene tubing introduced into a jugular vein Chlorisondamine (Ecolid R Ciba) was used to obtain a ganglionic blockade and was given at a dose of 0 2 to 0 3 mg/kg L-noradrenaline and adrenaline were given as an initial injection of 3 μg/kg followed by a continuous infusion of 0 5 μg/kg/min Adrenaline was also injected and infused at a fifth of the above dosage and rate

Urine was collected when normally voided and urinary content of adrenaline and noradrenaline was estimated fluorimetrically according to the procedure described by Euler and Lishajko (1961) Radioiodine (I^{131}) was used to study thyroid activity by methods previously reported (c f Andersson et al 1963) Blood glucose was determined enzymatically as described by Levin and Linde (1962)

Results

The response of normal goats to the ganglionic blocking agent

Before studying the effect of chlorisondamine on the thermoregulatory response to central cooling the drug was given i v to normal goats at the same dosages that were later used in thermode bearing goats When not present at the time of injection of the drug peripheral vasodilatation (as indicated by a rise in ear surface temperature) appeared within 5 to 10 min At this stage mydriasis started to develop and minor symptoms of bronchial congestion (intermittent coughing) appeared The peripheral vasodilatation remained for about 3 hours during which time the rectal temperature of the goats fell 0 5 to 1 5 C

2 *Response to local cooling of the heat loss center*

A) *Central cooling without ganglionic blockade* In both goats central cooling caused strong peripheral vasoconstriction within a few minutes At the onset of central cooling rather strong shivering was induced in the goat Andrew This shivering response gradually disappeared during the first hour of cooling No shivering was observed under the same conditions in the goat Cassius During two hours of preoptic/anterior hypothalamic cooling the rectal temperature of Andrew rose 1 5 C and that of Cassius 2° C A continuation of central cooling caused no further rise but the body temperature remained at the same high level until cooling was stopped In both goats local cooling of the heat loss center caused the expected marked thyroid activation (An

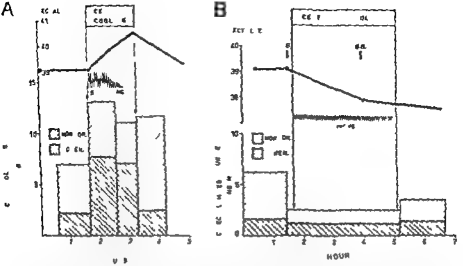

Fig 1 *The re ponse to local coolin of th "heat loss center in the goat Andrew befo e (A) and after (B) administration of a gan_l onic blockin_ agent*
Note the continued fall in rectal temperature the diminished excretion of catecholamines and the reduced shivering response after administration of the ganglionic blocking age it (chlorisondamine)
GB = I v inject ons of chlorisondamine (Ecolid Ciba 0.2 and 0 1 mg/kg)

dersson et al 1963) Urinary excretion of adrenaline and noradrenaline was determined in two experiments involving central cooling without ganglionic blockade in "Andrew During the cooling period the rate of adrenaline ex cretion rose 3 to 4 fold and noradrenaline excretion also increased (Fig 1 A) The blood glucose levels during three hours of central cooling gradually in creased by about 30 % in Andrew and by 20 % in "Cassius

B) *Central cooling during ganglionic blockade* Local cooling of the heat loss center was started 10 to 20 min after the administration of chlorisondamine At this time the rectal temperature of the goats had already started to decline and continued to do so also during the actual cooling period In some experi ments however the fall in rectal temperature was less pronounced during central cooling than it had been after administration of the ganglionic blocking agent alone The shivering response to central cooling in Andrew was much reduced during the ganglionic blockade and urinary excretion of sympathetic amines remained lower than before the administration of chlorisondamine even during the period of central cooling (Fig 1 B) In spite of the local cooling of the "heat loss center marked peripheral vasodilatation persisted at least for the first 15 min of central cooling and often much longer Further central cooling no longer caused a change in blood glucose level after the ganglionic blocking agent had been given but the degree of thyroid activation was the same as without pre treatment with chlorisondamine

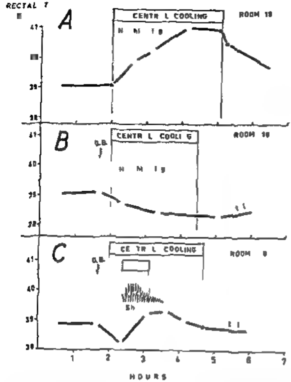

Fig [illegible] The response to local coolin of the heat loss center in the goat Cassius before (A) and after the administration of a ganglionic blocking agent (B) and the restoration of the response to central cooling by l noradrenaline infusion during ganglionic blockade (C)

Note that central cooling *per se* did not induce shivering in Cassius at this room temperature (18 C) (A and B) but did so during the infusion of noradrenaline (C)

GB = I v injection of the ganglionic blocking agent (chlorisondamine 0 3 mg/kg)

NA = Period of noradrenaline infusion (initial injections of 3 µg kg followed by infusion at a rat of 0 5 µg/kg/min)

The ganglionic blocking agent apparently increased the blood flow through the hypothalamus since it was necessary to increase the rate of cold water perfusion of the thermodes to obtain the same degree of local brain cooling as in corresponding experiments without previous administration of chlorisondamine

Fig 1 shows a comparison between the response to local cooling of the heat loss center in the goat Andrew before (A) and after (B) administration of chlorisondamine

3 Restoration of the response to central cooling by infusion of sympathetic amines during the ganglionic blockade

The effects of i v infusions of noradrenaline and adrenaline were studied in the goat Cassius during local cooling of the heat loss center after the administration of chlorisondamine Cassius was never observed to shiver during simple central cooling at this room temperature (18 C) or during similar cooling after the administration of chlorisondamine In contrast within a minute after the start of noradrenaline infusion the animal started to shiver Strong shivering lasted for a period of about 10 min but then gradually diminished Weak shivering outlasted the noradrenaline infusion by about 20

min (Fig 2 C) Intense peripheral vasoconstriction occurred soon after start of infusion while rectal temperature ceased to decline and started to rise It gradually rose 1° C during a one hour period of noradrenaline infusion (Fig 2 C)

The administration of noradrenaline apparently reduced hypothalamic blood flow since shortly after the onset of infusion there occurred a moderate drop of temperature 2 mm lateral to the cooling thermode The rate of cold water perfusion of the thermode therefore had to be slightly reduced to maintain a constant degree of cooling of the heat loss center during the noradrenaline infusion

Fig 2 shows the effect of cooling of the heat loss center in goat Cassius before (A) and after (B) the administration of chlorisondamine and the restoration of the response to central cooling by noradrenaline infusion during the ganglionic blockade (C)

The infusion of adrenaline had largely the same effect as that of noradrenaline The dosage first used was one fifth of that of noradrenaline since urinary excretion of adrenaline was generally found to be 3 to 5 times smaller than that of noradrenaline in goats under normal conditions (Andersson et al 1964) The infusion of this small amount of adrenaline during local cooling of the heat loss center performed under ganglionic blockade converted the fall in rectal temperature into a moderate rise but did not induce visible shivering It was therefore decided to increase the dosage of adrenaline to that of noradrenaline (see methods) In these circumstances adrenaline induced shivering although this was somewhat weaker than shivering observed during noradrenaline infusion The rise in rectal temperature was also somewhat smaller and it was noticeable that unlike the noradrenaline infusion the adrenaline initially caused an accentuation of peripheral vasodilatation Recordings of hypothalamic temperature revealed that the larger dose of adrenaline affected hypothalamic blood flow in the same manner as noradrenaline during the ganglionic blockade i e caused a decrease in flow

Discussion

The original observation of Cannon et al (1927) of increased adrenaline secretion in response to cold stimuli led them to suggest that increased non shivering heat production in the cold may be due to the calorigenic effect of adrenaline Since then the response of the sympathico-adrenomedullary system to acute cold exposure and during cold acclimation has been extensively studied (c f Euler 1961) Hsieh Carlson and Gray 1957 found that the ganglionic blocking agent hexamethonium eliminates the calorigenic response to cold in curarized cold acclimated rats This effect of hexamethonium was counteracted more effectively by the injection of noradrenaline than by the injection of adrenaline Additional information on the importance of noradrena

line and adrenaline in acute and chronic cold defence has recently been provided by Leduc (1961). His results also suggest that noradrenaline is the primary stimulant of non-shivering heat production and that adrenaline is important more as a supplementary defence against cold. Leduc's extensive studies involved the use of both adrenergic (phenoxybenzamine) and ganglionic (mecamylamine) blocking agents. It was found that the colonic temperature of non-acclimated rats treated with the adrenergic blocking agent remained normal at room temperature but fell in the cold. Similarly treated cold acclimated rats, however, could maintain normal body temperature for a few days in the cold. In contrast, both warm and cold adapted rats receiving the ganglionic blocking agent became moderately hypothermic at room temperature and rapidly died in severe hypothermia when exposed to cold. Sympathetic amines had a protective effect against this hypothermia, noradrenaline being more effective in cold adapted rats whereas adrenaline was more effective in warm adapted rats.

Previous studies in the goat have shown that local cooling of the preoptic/anterior hypothalamic region (the "heat loss center" of Magoun et al. 1938) causes marked thyroid activation (Andersson et al. 1963) and an increased production of sympathetic amines, especially of adrenaline (Andersson et al. 1964). In these studies it was also shown that the corresponding warming inhibits these hormonal cold defence mechanisms. As could be expected from previous studies in rats (Schapiro 1958, Leduc 1961) the administration of an adrenergic blocking agent (phenoxybenzamine) to the goat caused conspicuous increase in urinary excretion of catecholamines but did not cause hypothermia at room temperature. Further, the adrenergic blocking agent did not prevent the development of considerable hyperthermia during local cooling of the "heat loss center", even though the central cooling under these circumstances did not intensify peripheral vasoconstriction. A very marked increase in the shivering response to central cooling during the adrenergic blockade apparently contributed to the development of this hyperthermia (Andersson et al. 1964).

The present series of experiments shows that a ganglionic blocking agent prevents the development of hyperthermia during local cooling of the "heat loss center" and reduces the shivering response to such cooling. Fundamental differences thus seem to exist between the effect of adrenergic and ganglionic blocking agents in the defence against cold. It seems as if a response of the peripheral effector cells to adrenergic sympathetic impulses is not essential for an acute cold defence, but that the presence of a normal or even elevated level of circulating sympathetic amines is needed. The increased thermogenesis observed due to local cooling of the "heat loss center" under adrenergic blockade (Andersson et al. 1964) and in the present experiments due to similar cooling during ganglionic blockade combined with an infusion of sympathetic amines may partly be due to the calorigenic action of these amines but also to

the apparent facilitatory action of noradrenaline and adrenaline on the shivering mechanism Differences in the propensity of goats to shiver during simple central cooling (e g "Andrew" vs "Cassius") may in turn represent differential sensitivity of central structures to such facilitatory factors as circulating levels of sympathetic amines

From these experiments it may be concluded that an activation of the sympathico-adrenomedullary system is the primary cause of the temperature rise observed during local cooling of the "heat loss center". The thyroid activation *per se* does not seem to be of major importance since it was present to its full extent when the temperature rise was completely inhibited by the administration of the ganglionic blocking agent

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Reflex Influence of Mesenteric Afferents on Renal, Intestinal and Muscle Blood Flow and on Intestinal Motility

By

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Abstract

JOHANSSON, B. and J. B. LANGSTON. *Reflex influence of mesenteric afferents on renal, intestinal and muscle blood flow and on intestinal motility.* Acta physiol. scand. 1964. *61.* 400—412. — Stimulation of high threshold afferents in mesenteric nerves causes a reflex rise in blood pressure. Depressor effects are obtained at lower stimulus intensities. The autonomic response pattern reflexly induced by the mesenteric "pressor afferents" has been analysed in some detail. The blood pressure rise was found to be associated with a pronounced reflex vasoconstriction in the kidneys while vascular resistance in the skeletal muscles often decreased. Intestinal vessels constricted to a moderate extent and there was a profound inhibition of intestinal tone and rhythmicity. These reflex effects, including the intestinal inhibition, were all abolished by spinal anesthesia indicating that synaptic mechanisms in decentralized sympathetic ganglia are alone insufficient for mediation of the responses. The organization of the intestino-intestinal inhibitory reflex is discussed.

Some recent publications from this department have been concerned with the response patterns of cardiovascular reflexes, i.e. with the relative engagement of the heart and of the different vascular beds and their consecutive sections in certain reflex adjustments of the circulation. The reflex responses to alterations in baro- and chemoreceptor activity were studied with special regard to the blood flow changes in different vascular regions (muscle, kidney, intestine and skin) (Löfving 1961 a, b, Folkow, Johansson and Löfving 1961). A decrease in *baroreceptor* and/or *an increase in chemoreceptor activity* were found to induce the most pronounced vasoconstriction within the skeletal muscles while the reflex constrictions of the cutaneous and intestinal vessels were less prominent. Reactions of the renal vessels during baroreceptor adjustments were insignifi

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cant or absent as previously shown by Hartmann Orskov and Rein (1937) The vasoconstrictor fibres supplied to the various vascular regions seemed thus to be activated in a quantitatively differentiated manner

A separate study was devoted to the pressor and depressor response patterns initiated by afferent fibre systems in somatic nerves (Johansson 1962) Group III fibres predominantly from skeletal muscles were found to induce a depressor response due to a generalized inhibition of tonic activity in vasoconstrictor and accelerans fibres and to activation of vagal heart fibres Stimulation of afferent C-fibres mainly of cutaneous origin produced on the other hand a pressor response with a characteristic pattern of vasoconstrictor fibre activation causing a much more pronounced constriction of renal vessels than of skeletal muscle vessels Therefore this pressor response pattern differed considerably from that produced by carotid occlusion

In view of these findings it was considered of interest to investigate in some detail the circulatory responses to stimulation of visceral afferents also The present report is concerned mainly with regional vascular responses induced from pressor afferents originating in abdominal organs Special attention has been paid to blood flow changes in the kidney as compared to those in skeletal muscle Some experiments were designed to elucidate certain aspects on the nervous pathways mediating the reflex changes in blood flow and the accompanying reflex changes in intestinal motility

Methods

The experiments were performed on 45 cats usually anesthetized with chloralose 25–50 mg/kg after induction with ether However in some experiments on intestinal motility the animals were more deeply anesthetized with chloralose urethane (40–50 mg/kg and 80–100 mg/kg respectively) since intestinal tone and rhythmicity seemed to be better preserved with this type of anesthesia (cf Kock 1959) A tracheal cannula was inserted and the common carotid arteries as well as the vagal nerves were dissected in the neck Arterial blood pressure was recorded from one of the femoral arteries by means of an ordinary mercury manometer Heparine was given to prevent coagulation

Regional vascular responses were studied by diverting the venous outflow from the respective regions to a closed drop chamber where blood flow was recorded by means of an optical drop counter operating an ordinate writer (Clementz and Ryberg 1949 Lindgren 1958)

The deep femoral vein was cannulated for recording of blood flow in skeletal muscle and a tight ligature was placed around the ankle to reduce the admixture of cutaneous blood The left renal vein was cannulated for measurement of renal blood flow as on this side double veins are less frequently encountered To avoid engorgement of the kidney during the insertion of the cannula the renal artery was temporarily occluded after careful dissection near the aorta This can be done without damaging the renal vasomotor nerves

A section of the upper part of the jejunum was chosen for studying intestinal circulation and or intestinal motility The distal parts of the intestinal tract were extirpated The superior mesenteric vein was cannulated for intestinal blood flow recording To study changes in intestinal tone and motility a glass cannula was inserted into the

lumen of the gut and a small reservoir was connected to the cannula after the system had been filled with saline The intraluminal pressure in the jejunal loop was kept at 5–12 cm H_2O A volume recorder was connected to the reservoir

Reflex changes in regional blood flows are more easily evaluated if the arterial inflow pressure to the vascular beds studied is kept constant so that the recorded shifts in flow are more directly related to the neurogenic change in vascular resistance Arterial inflow pressure was often kept constant during reflexly induced blood pressure changes by adjustment of a screw clamp around the proximal abdominal aorta The left adrenal gland was denervated and the right one extirpated in order to eliminate the vascular effects of reflexly released adrenomedullary hormones In some experiments both adrenals were tied off and adrenocortical substitution was given by intramuscular injection of hydrocortisone (5 mg/kg)

Thin nerve bundles running along the branches of the superior mesenteric artery to intestinal parts that had been extirpated were prepared for stimulation in the afferent direction When intestinal blood flow or motility was not recorded nerve bundles along the main trunk of the mesenteric artery were dissected free for afferent stimulation Bipolar silver electrodes were used and the stimuli were delivered by a Grass Stimulator *via* an isolation unit

To avoid disturbances of the cardiovascular phenomena caused by reflex changes in respiration or skeletal muscle tone the animals were curarized with gallamine (Flaxedil) Artifical respiration was given by means of an adjustable pump set at appropriate rate and volume Gallamine was found often to depress intestinal tone and motility and was therefore excluded in many experiments where these parameters were studied

Some experiments were designed to elucidate the possible importance of reflex connections in decentralized abdominal sympathetic ganglia for mediation of some of the autonomic responses to stimulation of mesenteric afferents Complete preganglionic sympathetic blockade was produced during the course of the experiments by means of a spinal anesthesia This was accomplished by subdural administration of 1 5 2 ml of a 2 solution of lidocaine (Xylocain) *via* a thin polyethylene catheter which had been inserted through a lumbar or cervical laminectomy The tip of the catheter was at the lower thoracic level

Results

The reflex circulatory responses obtained by afferent electrical stimulation of mesenteric nerves were dependent upon the characteristics of the stimuli When the stimulus strength was gradually increased from low values a depressor response appeared at 2–5 V at a pulse duration of 1 msec A significant fall in blood pressure was obtained even at low frequencies of mesenteric nerve stimulation Afferent fibres with a considerably higher activation threshold (10–12 V at 1–2 msec) were found to produce pressor responses At low impulse rates the blood pressure rises were well sustained but of a moderate amplitude (10–30 mm Hg) while higher frequencies (above 20 sec) gave rise to initially pronounced pressure elevations which often subsided gradually Similar reflex pressor responses could be induced by natural stimuli like distension of an intestinal segment pinching of the peritoneum etc

Fig 1 illustrates the reflex blood flow changes in muscle and kidney when mesenteric pressor afferents were stimulated and also when the reflex pressor response caused by carotid occlusion was induced The animal was curarized

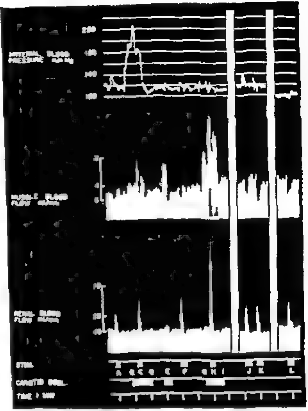

Fig 1 Cat 1 9 kg Chloralose Effects of stimulation of mesenteric pressor afferents and of carotid artery occlusion on blood pressure muscle and renal blood flow

A C F H J K and L Mesenteric nerve stimulation with 20 impulses/sec 2 msec and 12 V

B—D E and G—I Bilateral occlusion of the common carotid arteries

Arterial inflow pressure is kept constant in E—I and in K and L Atropine 1 mg/kg was given between K and L

Carotid occlusion produces the most pronounced vasoconstriction in the skeletal muscle region (E) while stimulation of mesenteric afferents gives a marked renal vasoconstriction accompanied by muscle vasodilatation (F J and K) The latter response is not significantly affected by atropinization (cf K and L) Mesenteric nerve stimulation during periods of carotid occlusion induces vasoconstriction in both kidney and muscle

The animal was curarized The vagal nerves were cut in the neck The right adrenal gland was tied off and the left one denervated Kidney weight 9 g

the adrenal medullae were inactivated as described and the vagal nerves were cut in the neck The effect of stimulation of the mesenteric nerves with 20 imp/sec 12 V and 2 msec is shown in A of Fig 1 A small rise in blood pressure is obtained and this is accompanied by a reduced renal blood flow and a slight increase in venous outflow from skeletal muscle Bilateral occlusion of the common carotid arteries (B—D) produces a more pronounced pressor response This is associated with a markedly reduced venous outflow from the skeletal muscles while there is no apparent change in renal blood flow Stimulation of the mesenteric afferents during the period of carotid occlusion (C) causes a reduction in venous outflow from both regions

The extent of the neurogenic changes in blood flow resistance in the two tissues are more clearly shown in the subsequent part of Fig 1 as here the arterial inflow pressure was kept constant during the reflex adjustments Carotid occlusion (E) is seen to produce a marked increase in the vascular resistance within the skeletal muscles while only a slight reflex vasoconstriction is observed in the kidney In the reflex pressor response to mesenteric nerve stimulation (F) on the other hand flow resistance is markedly increased in the kidney but reduced

Fig 2 Cat 2 5 kg Chloralose urethane Effects of stimulation of mesenteric afferents and splanchnic nerve efferents on blood pressure intestinal motility and intestinal blood flow

A C and E Stimulation of mesenteric afferents with 2 4 and 8 imp/sec respectively at 12 V and 2 msec

B D and F Stimulation of the peripheral end of the left splanchnic nerve with 4 8 and 16 imp/sec respectively at 12 V and 2 msec

Mesenteric nerve stimulation produces inhibition of intestinal tone and motility even at low impulse rates Stimulation of splanchnic efferents at 4 imp sec does not cause intestinal inhibition while at 8 imp/sec a sluggish response is obtained. The effects of mesenteric and splanchnic nerve stimulation on intestinal blood flow are significantly different

The vagal nerves were cut in the neck and both adrenal glands were tied off

in the skeletal muscles This latter pattern is seen to be modified if the mesenteric afferents are stimulated H) during a prolonged occlusion of the carotid arteries from G to I) The renal vasoconstrictor response to stimulation of the visceral afferents is augmented by a temporary unloading of the baroreceptors while the reaction of the muscle vessels is changed from vasodilation (F) to vasoconstriction H)

The muscle vasodilatation observed in F can be explained theoretically as due either to a reflex activation of the cholinergic vasodilator fibres supplied to this region or to an inhibition of its tonic vasoconstrictor fibre activity Both mechanisms may be active but the latter one is at least partly responsible since the vascular response in the skeletal muscles obtained after atropinization (L) is not significantly different from that before J and K)

The reflex influence of the mesenteric pressor afferents on the intestinal vessels and on the smooth muscles of the intestinal wall was studied in experiments with recordings of blood flow and motility in the proximal part of the jejunum When mesenteric nerve bundles originating in more distal parts of the gut were stimulated in the afferent direction with high stimulus intensities reflex

intestinal vasoconstriction and prompt reflex inhibition of intestinal tone and motility were found to accompany the induced rise in blood pressure

Fig 2 illustrates a simultaneous recording of arterial blood pressure intestinal motility and intestinal blood flow in an experiment where both adrenal glands had been tied off with ligatures and the vagal nerves cut in the neck The left splanchnic nerves were cut and the distal end prepared for stimulation (see below) while the right splanchnics were intact A C and E of Fig 2 represent stimulations of mesenteric afferents with increasing impulse frequencies (2 4 and 8 imp/sec respectively) The reflex effects are seen to occur even at these low frequencies Fig 2 further illustrates the quantitative difference between this reflex response pattern induced from the mesenteric afferents and that obtained by direct stimulation *of the efferent preganglionic fibres in the splanchnic* nerves In B D and F of Fig 2 the left splanchnics were stimulated with 4 8 and 16 imp/sec respectively The degree of vasoconstriction induced by efferent stimulation is gradually increased by increasing the impulse frequency A significant inhibition of intestinal tone and rhythmicity is obtained only at relatively high impulse rates (D F) but not in the low frequency range (B) Thus in contrast to what was seen at stimulation of mesenteric afferents low frequency activation of the splanchnic preganglionic efferent fibres has no significant effect on intestinal motility (provided that the release of adrenomedullary hormones is prevented)

The present findings concerning the effects of preganglionic sympathetic stimulation on intestinal motility are in agreement with previous observations by Celander (1959) and Kock (1959) They found that efferent stimulation of the splanchnic nerves in adrenalectomized animals produced inhibition of intestinal *motility only at impulse frequencies higher than those seen in sympathetic* efferents under physiological conditions The intestinal inhibition thus obtained was attributed to the reduced nutritional blood flow caused by the pronounced vasoconstriction or to local overflow of adrenergic transmitter from the vasoconstrictor end organs to the smooth muscles of the intestinal wall In view of his findings Celander questioned the existence of true intestino-inhibitory fibres in the preganglionic sympathetic outflow

The prompt and profound intestinal inhibition elicited from mesenteric afferents in A C and E of Fig 2 are accompanied by such moderate reductions in blood flow that they cannot reasonably be secondary to the reflex vasoconstrictor fibre response (cf A and B in Fig 2) They must be ascribed instead to reflex activation of true inhibitory fibres innervating the smooth muscles of the intestinal wall However if Celander is correct in saying that no such fibres exist in the preganglionic sympathetic nerves quite specialized reflex connections must be assumed for the mediation of these intestinal inhibitions In experiments made by Kuntz and van Buskirk 1941) and by Kuntz and Saccomanno (1944) reflex inhibition of motility produced in one segment of the gut by distension of another isolated segment was found to persist after preganglionic denervation

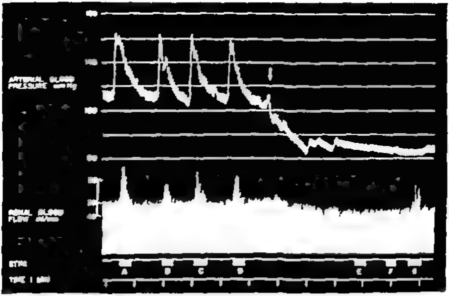

Fig 3 Cat 2 7 kg Chloralose Effect of spinal anesthesia on the renal vascular responses to mesenteric nerve stimulation
A—F Stimulation of mesenteric afferents with 30 imp/sec 10 V and 2 msec
G Stimulation of renal nerves with 3 imp sec 10 V and 2 msec
The renal vasoconstrictor response to mesenteric nerve stimulation is abolished by subdural injection of lidocaine given at arrow The irregularities of the blood pressure recording between D and E are due to dextrane transfusions
The animal was curarized and the vagal nerves were cut in the neck

Synaptic mechanisms in the mesenteric ganglia were assumed to mediate this intestino-intestinal inhibitory reflex There is histological (Kuntz 1938 1940) and neurophysiological (Job and Lundberg 1952 Brown and Pascoe 1952 Bessou Laporte and Planel 1959) evidence for the existence of synaptic connections in decentralized mesenteric ganglia In the experiments described below attempts have been made to find out whether such ganglionic reflex connections could possibly explain at least some of the autonomic responses to excitation of mesenteric pressor afferents shown in Fig 1 and 2 Not only the intestinal inhibitions as suggested by Kuntz and co-workers but also the reflex renal and intestinal vasoconstriction might be induced *via* such reflex arcs

Renal blood flow was recorded together with intestinal motility in 16 experiments in order to study the effects of mesenteric nerve stimulation before and after the induction of spinal anesthesia The renal vasoconstrictor response was regularly and totally abolished after subdural administration of lidocaine A representative recording of renal blood flow from these experiments is shown in Fig 3 In A B C and D a reproducible reflex vasoconstriction is produced in the kidney by stimulation of nerve bundles along the superior mesenteric artery After spinal anesthesia induced after D the reflex changes in blood pressure and in renal blood flow are no longer obtained (E and F) The elimina

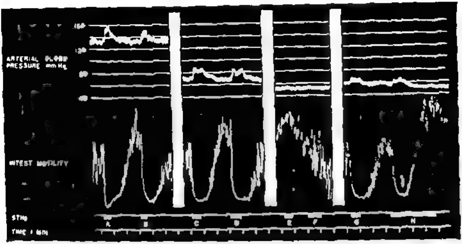

Fig 4 Cat 2 ? kg Chloralose urethane Effect of cord transection and spinal anesthes a on the intestinal inhibitory response to st mulation of mesenteric afferents

A—H Afferent stimulat on of mesenteric nerve branches vith 10 imp/sec 12 V and 2 msec Total transection of the cervical spinal cord (between B and C) does not affect the intestinal response while subdural inj ction of lidoca ne (between E and F) eliminates the inhibitory effect of the stimulation The response can be elicited again after the spinal anesthesia has faded off (G) The effect of a prolonged mesenteric nerve stimulation (H) is broken by another subdural injection of lido a ne (arrow)

The vagal nerves were cut in the neck

tion of the renal vasoconstriction cannot be attributed to any reduced respon siveness of renal vessels to vasoconstrictor fibre activity since direct low fre quency stimulation of nerve branches running to the kidney (G) causes a reduc tion of renal blood flow No support has therefore been obtained for the hypothesis that nervous connections in decentralized sympathetic ganglia should be of functional importance for the mediation of renal va oconstriction in response to activation of mesenteric afferents This is apparently true also for the intestinal vasoconstriction

In view of the previous findings of Kuntz and co workers referred to above the intestinal inhibitory response to mesenteric nerve stimulation was expected to persist after induction of spinal anesthesia However the intestin testinal inhibitory reflex seemed like the renal vasoconstrictor response to lished after subdural injection of lidocaine This was more clearly shown aral series of experiments on chloralose urethane anesthetized animals we not given gallamine The results obtained in one of these experi trated in Fig 4 Stimulation of mesenteric afferents is een to to produce a rise in blood pressure and a characteristic inhil. ... tone and rhythmic activity (cf A C and E of Fig 2) Artificial then administered and after transection of the pinal cord at t the intestinal response was found to be unchanged (C and D) that higher centres of the CNS are not required for mediation

tween D and E lidocaine was injected into the subdural space *via* a catheter with its tip at the lower thoracic level Mesenteric nerve stimulation was afterwards without any effect on intestinal motility (E and F) and circulatory and somatomotor reflex responses were abolished as well 40 minutes later the spinal anesthesia had faded off and mesenteric nerve stimulation was again able to produce intestinal inhibition rise in blood pressure and reflex contractions in the muscles of the abdominal wall (G) Another subdural injection of lidocaine given at arrow during a prolonged period of mesenteric nerve stimulation (H) is seen to interrupt the reflex intestinal inhibition and the blood pressure rise These results indicate that an effective spinal anesthesia which abolishes circulatory and somatomotor responses to mesenteric nerve stimulation will eliminate also the intestino-intestinal inhibitory reflex

Comments

Afferent stimulation of mesenteric nerve bundles at moderate intensities produced a reflex fall in blood pressure The afferent fibres responsible for this effect may be functionally related to those described in man by Folkow et al (1962) Their stimulus threshold corresponds well with that of somatic depressor afferents (Johansson 1962) Part of them might mediate impulses from mesenteric baroreceptors (Sarnoff and Yamada 1959)

In the present investigation the main attention was paid to the circulatory r pattern induced from high threshold afferents in the mesenteric nerves be results illustrated in Fig 1 demonstrate clearly the difference in the patterns of vasoconstrictor fibre response produced by these visceral pressor fibres and by carotid occlusion The alterations in baro-chemoreceptor activity produced by the latter procedure affect renal vasoconstrictor fibre discharge to a relatively slight extent which is in agreement with the observations by Lofving (1962 a b) referred to above In contrast visceral pressor afferents originating in gut or mesentery exert a pronounced excitatory action on renal vasoconstrictor neurones as judged from the pronounced vascular responses induced in the kidney Central structures related to muscle vasoconstrictor fibres are on the other hand comparatively more influenced by the cardiovascular baro- and chemoreceptors than by mesenteric pressor afferents The vasodilatation obtained in muscle in A F J K and L of Fig 1 are at least partly due to inhibition of tonic vasoconstrictor fibre activity This inhibition may be secondary to an increased baroreceptor discharge in association with the rise in carotid and aortic blood pressure caused by the mesenteric nerve stimulation This interpretation is supported by the appearance of a weak but significant vasoconstriction in the skeletal muscles when the visceral afferents were stimulated under circumstances where the buffering capacity of the baroreceptors had been reduced by carotid occlusion C and H in Fig 1) The mesenteric pressor afferents have thus been shown to exert an excitatory action on the vasoconstrictor fibres

especially on those to the kidneys and intestine but only to a slight extent on those to the skeletal muscles Since the reflex influence on the vasoconstrictor fibres of the latter tissue is so weak it is easily overcome by the inhibitory effect of a secondary baroreceptor activation

The reported findings illustrate the complexities that exist in the interaction of certain cardiovascular reflexes Quantitative differences in the excitability of the different central neurone pools and in the extent of afferent fibre convergence upon them may create considerable potentialities for differentiation of vasomotor reactions and may thus explain the often complex response patterns

It is notable that the mesenteric pressor afferents produce a circulatory response which is closely similar to that elicited by stimulation of somatic C-fibres of cutaneous origin (Johansson 1962) It also resembles in some respects the cardiovascular adjustment which accompanies the defence alarm reaction initiated from its reflex centre in the hypothalamus which is known to be easily triggered by painful stimuli (Abrahams Hilton and Zbrozyna 1960) Reflex blood pressure rises could be produced in the present experiments by pinching or distension of abdominal structures Such procedures would apparently be painful to a conscious subject and it is possible therefore that the pressor responses studied represent a nociceptive cardiovascular reflex. It is true that deep pain or pain from visceral organs often produces an opposite type of cardiovascular reaction characterized by bradycardia and fall in blood pressure (Folkow et al 1962 Johansson 1962) but this depressor reflex is related to afferent fibres with a relatively low threshold to electrical stimulation Nociceptive impulses of abdominal origin is possibly mediated by two different sets of afferent fibres which differ somewhat with respect to their "adequate" stimuli and whose reflex influences on the circulatory system are of opposite directions

The observations of renal and intestinal vasoconstriction in response to mesenteric "pressor fibre" stimulation suggested that quite specific nervous connections might exist between the afferent fibre system and the renal and intestinal vasoconstrictor efferents As mentioned above peripheral reflex connections within the abdominal sympathetic ganglia have repeatedly been suggested to be of importance for mediation of intestinal inhibition in response to stimulation of mesenteric afferents Therefore the possible existence and significance of such ganglionic synaptic mechanisms for mediation of the renal and intestinal vasoconstriction could not *a priori* be excluded The present results indicate however that the reflex vasomotor responses to mesenteric nerve stimulation and in fact also the accompanying reflex changes in intestinal motility are dependent upon central nervous connections and cannot be elicited *via* reflex mechanisms in the decentralized ganglia The present observations with bearing on the organization of the intestino-intestinal inhibitory reflex are therefore somewhat confusing in the light of previous studies The neurophysiological investigations by Job and Lundberg (1952) by Brown and Pascoe 1952) and by Lessou Laporte and Planel (1959) demonstrated the existence of reflex connections in

decentralized mesenteric ganglia It has not been possible however, to prove their functional relationship to the intestino intestinal inhibitory reflex The present results indicate that such peripheral mechanisms are alone insufficient for the production of this response This is contradictory to the findings of Kuntz and van Buskirk (1941) and Kuntz and Saccomanno (1944) but in agreement with those of Freund and Sheehan (1943) The latter investigators were unable to elicit intestino-intestinal inhibition in cats which had previously been subjected to total thoraco-abdominal preganglionic sympathectomy and they concluded that the reflex must be mediated *via* the central nervous system Furthermore Chang and Hsu (1942) found in experiments on dogs that transection of thoraco-lumbar dorsal roots eliminated the intestino-inhibitory response and they considered the reflex centre to be located in the Th6—L2 segments of the cord The most probable explanation for the diverging results of Kuntz and co-workers seems to be that the preganglionic denervation may have been incomplete in their experiments

It is evident from Fig 2 above that the changes in intestinal motility and blood flow which are produced by preganglionic stimulation are quantitatively very different from those which are reflexly induced by afferent stimulation distal to the ganglion In experiments on colonic blood flow and motility Oscarsson (1955) found no such difference in the response to preganglionic and to peripheral afferent stimulation of fibres to the inferior mesenteric ganglion but he used relatively high impulse frequencies He concluded from these and from certain neurophysiological findings that the two presynaptic fibre systems had an extensive convergence on the postganglionic cells The great majority of the colonic nerve neurons could be discharged from either of the two presynaptic systems The degree of occlusion was estimated to 80—100 per cent

The significant differences between the response patterns evoked by low frequency stimulation of the splanchnic nerve and of the mesenteric nerve branches (Fig 2) indicate that there are two entirely different sets of efferent fibres innervating the smooth muscles of the intestinal wall and those of the intestinal vessels respectively The prompt inhibitory effects on motility and tone elicited from the mesenteric afferents even at low frequency stimulation must be considered to be mediated by true inhibitory efferents and cannot be merely secondary to a reduction in intestinal blood flow or to local overflow of adrenergic transmitter from vasoconstrictor nerve endings It has recently been shown histochemically that adrenergic nerve fibres are supplied to the myenteric plexa of the intestinal wall (Norberg 1963 personal communication) The intestino-inhibitory fibres seem to be exclusively associated with the intestino-intestinal inhibitory reflex and they are evidently not engaged in such sympatho-excitatory responses as produced for instance by carotid occlusion or by stimulation of somatic "pressor afferents Inhibition of intestinal motility in the latter types of reactions have been shown to be due to liberation of adrenomedullary hormones (Kock 1959)

Since the reflex centre for the intestino-intestinal inhibition seems to be in the CNS the anatomical pathway for the intestino inhibitory efferents from the spinal cord to the abdominal viscera remains to be demonstrated The abdominal sympathetic outflow seems to be the most probable location for this pathway but it has not been possible to demonstrate it unequivocally by direct splanchnic nerve stimulation (Celander 1959 Kock 1959 Fig 2 above) The stimulation frequencies at which intestinal inhibition may be induced from the splanchnic nerves are above the physiological discharge range of autonomic efferent systems and it seems unlikely that intestino inhibitory fibres should be exceptional in that respect the more so as the stimulation of the visceral afferents at only 1—4 impulses/sec produces a prompt reflex activation of the proposed efferent fibres Also the possibility that these fibres should be particularly resistant to electrical stimuli seems to be ruled out by the fact that splanchnic stimulation at very high intensities (30—50 V) — well above that required for maximal vasoconstrictor fibre activation — failed to cause intestinal inhibition in the present experiments It seems as if the efferent discharge pattern which is reflexly produced by the afferent impulses cannot be sufficiently well reproduced by artificial stimulation of the splanchnic nerves It is possible that direct electrical stimulation of intestino-inhibitory fibres in these nerves is unable to affect intestinal motility due to a simultaneous activation of some other efferent system which interferes in some way with the transmission of the intestino-inhibitory impulses in the ganglia or in the myenteric plexa This latter explanation is at present entirely speculative and its validity has to be tested in further experiments

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Accommodation of Single Myelinated Nerve Fibres from Xenopus Laevis Related to Type of End Organ

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Abstract

VALLBO, Å. B. *Accommodation of single myelinated nerve fibres from Xenopus laevis related to type of end organ.* Acta physiol. scand. 1964. *61.* 413—428. — Accommodation and repetitive firing of single nerve fibres were determined in excised preparations from the clawed toad. Sensory fibres innervating fast and slowly adapting mechanoreceptors and motor fibres were studied. It was found that accommodation was slightly faster in motor than in sensory nerve fibres, but a significant difference was not found between the two types of sensory nerve fibres in this respect. The receptors innervated by the single nerve fibres were stimulated with long mechanical pulses and the sensory discharge was compared with the repetitive activity due to electrical stimulation of the axons. The discharge elicited by the slowly adapting receptors was of longer duration and of wider impulse frequency range than the repetitive firing due to electrical stimulation of their axons. It was concluded that measurement of accommodation in peripheral nerve fibres does not give any indication of the mechanism for the variation of adaption among the mechanoreceptors studied. Further, it was concluded that receptor discharge cannot generally be explained by the hypothesis that propagated impulses are initiated by a maintained current depolarizing a nerve membrane which has the same properties as the membrane in the peripheral nerve without further assumptions. A slower accommodation at the site of impulse initiation and some mechanism modifying the impulse frequency must be assumed in the slowly adapting receptors to make the results of the present investigation consistent with this hypothesis.

It is generally held that the impulse in the afferent nerve fibre is initiated by a generator current depolarizing the nerve membrane (Bernhard, Granit and Skoglund 1942, Granit 1947). This view which has been supported by several investigations (for ref. see Gray 1959) requires that nerve fibres innervating the most slowly adapting receptors have almost no accommodation at the site of impulse initiation while accommodation at this site could be more pronounced in fibres innervating faster adapting sense organs, although the hypothesis does

not imply that the nerve fibre has the same properties at the site of impulse initiation as in the peripheral nerve some distance from the sense organ. It has not been possible to study the properties of the nerve membrane at the exact site of impulse initiation. On the other hand accommodation and repetitive firing of peripheral nerve fibres have been studied in many investigations and discussed in relation to the generator current hypothesis.

Repetitive firing to long electrical pulses may occur in mammalian (Skoglund 1942, Granit and Skoglund 1943), amphibian (Erlanger and Blair 1936, 1938, Katz 1936) and crustacean (Hodgkin 1948) nerve fibres. The maximum duration of repetitive activity in mammalian and crustacean nerve fibres is considerable and might possibly be as long as the maximum discharge duration of slowly adapting receptors. Repetitive activity of amphibian nerve fibres does not, however, seem to exceed a few tenths of a second. The amphibian experiments have been criticized because excised preparations have been used and accommodation might be unphysiological due to withdrawal from circulation (Parrack 1940, Skoglund 1942). The critical slope of the nerve fibre to electrical stimulation seems to be of the same order of magnitude as that of the corresponding sense organ to mechanical stimulation in case of fast adapting skin mechanoreceptors in the frog (Gray and Malcolm 1951) and Pacinian corpuscles in the cat (Gray and Matthews 1951). Further, the general discharge characteristics of the Pacinian corpuscles can be imitated by electrical step stimulation of their axons (Gray and Malcolm 1950). The impulse frequency of some of the mammalian and crustacean nerve fibres is variable over a wide range with the intensity of the stimulating pulse. Most of these findings seem to be in agreement with the statement that "there is no evidence or need to suppose that the part of the afferent fibre in which the impulses are set up differs from other parts of nerve fibres in its response to flow of current" (Gray 1959).

If the nerve fibre has the same properties throughout its length it follows that the maximum discharge duration when the nerve fibre is stimulated with a step current of long duration must not be shorter than the maximum discharge duration of the sense organ. It does not immediately follow from the above assumption that the range of impulse frequency in the nerve elicited through the sense organ would correspond to the range that is obtained on stimulation of the nerve. Several complications might disturb such a simple relation, e.g. a low safety factor at branching points in nerve fibres which ramify extensively at the receptor (Katz 1950).

In the present investigation the discharge of single mechanoreceptors was compared with the repetitive activity of their own axons. Fast and slowly adapting units were studied. A reasonable agreement was found between the maximum number of nerve impulses elicited by mechanical stimulation of the fast adapting receptors and electrical stimulation of the corresponding nerve fibres. The slowly adapting mechanoreceptors, on the other hand, gave a considerably longer discharge than the maximum repetitive activity elicited by electrical

stimulation of their axons There was also a pronounced difference with respect to impulse frequency range in the slowly adapting units due to the two modes of stimulation

Accommodation is faster in efferent than in afferent nerve fibres both in the cat (Skoglund 1942) and in the frog (Erlanger and Blair 1938) Granit and Skoglund (1943) compared accommodation of skin and muscle afferents (cat) and found that the former had almost no accommodation while the latter did accommodate to some extent In these studies the activity of one or several fibres has been recorded from nerve fibre bundles This method selects the fibres with slow accommodation (Cray and Matthews 1951) It seems therefore that this type of experiment allows the conclusion that there are fibres of slower accommodation among the skin afferents than among the muscle afferents but it cannot with certainty be stated whether nerve fibres innervating different types of end organs fall in distinct groups with respect to accommodation It seems necessary to measure accommodation of isolated nerve fibres to settle such a question If the accommodation of a nerve fibre has direct significance for the adaptation of the sense organ it seems likely that there would be a pronounced difference in accommodation between fibres innervating fast and slowly adapting receptors

In the present investigation the accommodation of single isolated toad nerve fibres of three types was measured motor fibres sensory fibres innervating fast adapting skin mechanoreceptors and sensory fibres innervating slowly adapting muscle mechanoreceptors It will be shown that accommodation was faster in motor than in sensory fibres although there was a considerable overlap No difference in accommodation was found between sensory nerve fibres innervating slowly adapting receptors and those innervating fast adapting receptors

Methods

Preparation Excised preparations from *Xenopus laevis* were used The preparation consisted of the foot and the sciatic tibial nerve Single myelinated nerve fibres were dissected from either of the two main branches of the tibial nerve The dissected fibre was kept intact with its end organ This made it possible to determine which type of end organ was innervated by the single fibre and also to study the discharge in the nerve fibre in response to mechanical stimulation of the attached mechanoreceptor

Stimulation of receptors Cutaneous receptors were mechanically stimulated with a Perspex rod with a flat surface of about half a square centimeter at the end This rod was fixed onto an electromagnet which was driven by rectangular pulses from an ordinary stimulator When the pulse was applied the end of the Perspex rod moved about 3 mm from one fixed position to another The shape of this mechanical pulse was recorded photoelectrically in one experiment The onset of the movement was rather slow but 90 of the movement occurred during 6 msec Damped oscillations were present for about 30 msec The maximum amplitude of the oscillations was about 250 μ and the frequency about 550 cps The intensity and the localization of the stimulation were adjusted to give approximately the maximum number of impulses from the receptor studied The foot was fixed with pins onto a cork plate during the stimulation The muscle

receptors were activated by extending the muscles *in situ* with a dropping weight attached to the toes with a thread. The amount of extension was not standardized. Positive evidence that a dissected fibre was of motor function was obtained by stimulating the single fibre electrically and observing a muscle twitch in response to this stimulation.

Stimulation of nerve fibres. The nerve fibres were stimulated with two different techniques. In about half the cases the fibres were stimulated at the dissected region with the technique described by Frankenhaeuser (1957). This technique implies that a single node was studied under conditions in which no current from the membrane was allowed to spread in the internodes and all the stimulating current passed through the investigated node. This technique will henceforth be called 'focal stimulation of one node'. In the other type of experiment the part of the nerve trunk between the end organs and the dissection of the single fibre was mounted in a Perspex cell with five 2–3 mm wide pools filled with Ringer's solution. Vaseline seals were applied over the nerve on the partitions to separate the fluid pools. The nerve was stimulated at one or several sites with Ag-AgCl electrodes dipping into the pools distally to the dissection of the single fibre and the conducted impulses were recorded from the nerve trunk proximal to the dissection. This technique of nerve stimulation will be referred to as 'bipolar stimulation on nerve trunk'. The nerve was routinely stimulated at a desheathed site but in some cases it was also stimulated through intact nerve sheath. When fibres from muscle mechanoreceptors were studied with this technique the nerve was blocked with local anaesthetic close to the muscle in order to avoid confusion from activation of intrafusal muscle fibres.

The critical slopes of the single nerve fibres were determined with linearly rising currents the slope of which was changed in steps of 10–20 %. The critical slope was defined as the minimum slope giving rise to an action potential. Rectangular current pulses of various intensities and long duration were applied and the response of the single nerve fibre was recorded to determine the characteristics of repetitive firing when this occurred. 'Repetitive firing' without further definition will henceforth be used to denote activity consisting of more than one action potential in a single fibre when stimulated with long electrical rectangular pulses.

Recording. The sensory discharge was recorded as conducted impulses from the nerve trunk proximal to the dissection across an air gap and in a few cases across a vaseline gap applied over the single nerve fibre. The same techniqeue was used for recording the activity elicited by bipolar electrical stimulation of the nerve fibres. When focal stimulation of one node was used the unattenuated membrane action potential of a single node was recorded according to the technique described by Frankenhaeuser (1957).

Solution. The preparations were kept in Ringer's solution of the following composition during the dissections and the experiments: NaCl 112.0, KCl 2.5, $CaCl_2$ 2.0, $NaHCO_3$ 2.5 (mM). The pH of this solution was 7.5–8.0.

The experiments were performed at room temperature (18–21° C) and the toads had been kept at about the same temperature for weeks before (Katz 1936).

Nomenclature. The term 'accommodation of nerves' has been used to denote a threshold rise at the cathode in consequence of a prolonged electrical stimulation. However, it has been shown that the threshold of a restricted membrane area, e.g. a node in a myelinated nerve fibre, is fairly constant regardless of the shape of the stimulating current (Tasaki 1950, Frankenhaeuser 1952), but that the amount of current produced by the membrane during activity varies smoothly with the slope of the stimulating current; the membrane current decreases as the slope of the stimulating current is decreased (Frankenhaeuser 1952). Accommodation will therefore be used to denote the change in nerve response to slowly rising currents. In the present investigation the rate of accommodation was determined by the critical slope, i.e. the minimum slope of linearly rising current which gave rise to a propagated impulse in case of bipolar stimulation and a clear

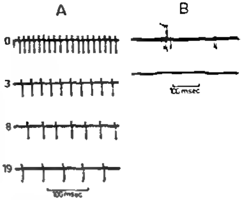

Fig 1 Discharge in the afferent nerve fibre in response to maintained mechanical stimulation of the two types of receptors studied Slowly adapting muscle mechanoreceptor (A) Figures at left indicate time in seconds after the first record which was taken about one second after the extension was applied Fast adapting skin mechanoreceptor (B) Lower trace indicate mechanical pulse

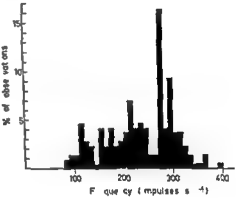

Fig 2 Histogram showing the distribution of mean nerve impulse frequencies in single fibres innervating fast adapting skin mechanoreceptors in response to stimulation of the sense organs with long mechanical pulses

action potential in case of focal stimulation of one node (cf Vallbo 1964) Adaptation will be used to signify the decline in nerve impulse frequency during maintained stimulation of the receptor The basis for classifying the sense organs as fast or slowly adapting was the time taken for adaption to zero frequency

Results

Discharge in single nerve fibres in response to mechanical stimulation of receptors

The discharge in nerve fibres from single skin and muscle mechanoreceptors due to maintained mechanical stimulation of the receptors was studied The cutaneous receptors gave a short burst of impulses during the first 10—30 msec of the stimulation and occasionally also a short off response (Fig 1 B Fig 7 C) The maximum number of impulses varied from one receptor to another between 3 and 11 (Fig 11 circles) The discharge frequency declined slightly towards the end of the burst in most cases The mean impulse frequencies of the bursts are shown in histogram in Fig 2 The percentage of total observations of

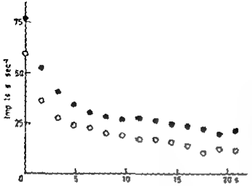

Fig 3 Two adaptation curves of a muscle mechanoreceptor Nerve impulse frequencies (ordinate) against time (abscissa) Zero time about one second after extension was applied

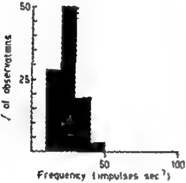

Fig 4 Histogram showing the distribution of nerve impulse frequencies in single nerve fibres innervating slowly adapting muscle mechanoreceptors in response to extension of the muscle Impulse frequency measured 6 sec after the extension was applied

the frequencies given by the abscissa in steps of 10 impulses/sec is shown by the height of the bars The histogram is based upon 215 observations from 13 receptors The mean impulse frequency varied roughly between 100 and 350 impulses/sec

The mechanoreceptors in the muscles on the other hand gave a long continuous discharge which was routinely recorded for 20—30 sec The impulse frequency declined with time towards a final low value The discharge at different times after applied stretch is shown in Fig 1 A Two adaptation curves from the same unit which is representative of all the muscle mechanoreceptors are shown in Fig 3 Maximum and minimum discharge frequencies seen from these receptors were 250 and 10 impulses/sec respectively This type of discharge is well known as characteristic for muscle spindles and Golgi tendon organs Fig 4 shows the discharge frequency from the muscle mechanoreceptors as measured 6 sec after the extension was applied The histogram is based upon 32 observations from 12 receptors These results show that there was a pronounced difference between the two types of receptors with respect to discharge duration as well as impulse frequency range

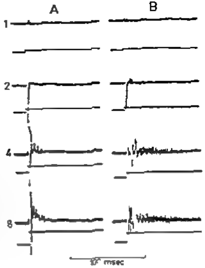

Fig 5 Repetitive activity of the muscular branch of the tibial nerve when stimulated with long step currents at a desheathed site (A) and through intact nerve sheath (B) Figures on the left hand side of the records give the intensities of the stimulating pulses in units of rheobases. Stimulating current is also indicated by the lower trace Retouched

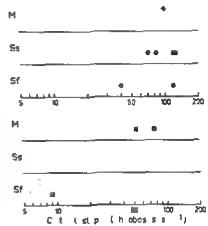

Fig 6 Critical slopes of single nerve fibres innervating three different types of end organs motor fibres (M) sensory fibres innervating slowly adapting muscle mechanoreceptors (Ss) and sensory fibres innervating fast adapting skin mechanoreceptors (Sf) The upper half of the figure shows the results obtained by bipolar stimulation on nerve trunk and the lower half those obtained by focal stimulation of one node (see Methods)

Repetitive firing of nerve fibres due to electrical stimulation at desheathed sites and through intact nerve sheath

Before presenting the complete data concerning accommodation and repetitive firing of nerve fibres it is convenient briefly to report one finding which was not systematically investigated but which must be taken into account in the following It was found that the critical slope and the duration of the repetitive firing were somewhat dependent upon whether the nerve sheath was intact at the site of stimulation or not The critical slope was lower and the repetitive firing was more pronounced when the nerve sheath was intact This is illustrated in Fig 7 and 8 In both these figures A shows the activity of a single fibre in response to step current stimulation at a desheathed site and B shows the activity of the same fibre in response to stimulation through intact nerve sheath Qualitatively the same differences were observed in several single nerve fibres innervating

Table I Probability levels of the differences in critical slopes between nerve fibres innervating different types of end organs

M	vs	S	$0.01>P>0.001$	Significant
M	vs	[illegible]	$0.05>P>0.02$	Significant
M	vs	Sf	$0.01>P>0.001$	Significant
Sf	vs	Ss	$P>0.1$	Insignificant

M denotes motor fibres, Ss sensory fibres innervating slowly adapting muscle mechanoreceptors, Sf sensory fibres innervating fast adapting skin mechanoreceptors and [illegible] sensory nerve fibres, i.e. Ss and Sf taken together

all three types of end organs. It is possible that desheathing might have damaged some of the superficial fibres in the trunk but it seems unlikely that the more centrally placed fibres could have suffered from this procedure. In order to check if the difference in repetitive activity of single nerve fibres with intact and removed connective tissue sheath might be due to injury the response of the whole nerve trunk was recorded in five experiments. The difference between stimulation at a desheathed part of the nerve and through intact sheath is illustrated in Fig. 5. It can be seen that maximum repetition duration was considerably less when the nerve was stimulated at a desheathed site (A). This suggests that there was an overall change in accommodation when the nerve sheath was removed. A decrease in repetitive firing in excised mammalian nerves after desheathing has been described by Adrian (1930) and Erlanger and Blair (1936).

The maximum duration of repetitive activity observed from the whole nerve with intact sheath was roughly 150 msec. The recording conditions were the same in these experiments as in the single fibre experiments and it seems therefore justified to conclude that the maximum duration of repetitive activity of the single fibres as studied here did not exceed 150 msec. About the same duration was seen in one single nerve fibre (Fig. 8 B).

Accommodation in single nerve fibres

The critical slopes of single nerve fibres were determined. Motor fibres and sensory fibres innervating fast and slowly adapting mechanoreceptors were studied. The findings are illustrated in Fig. 6. The critical slopes in rheobases/sec are given by the scale. Each point represents one nerve fibre. The letters to the left indicate the type of end organ. The results from bipolar stimulation on nerve trunk are shown at the top (circles). The stimulation was applied at desheathed sites of the nerve in all these cases. The results obtained by focal stimulation of one node are shown at the bottom (squares).

It is seen from Fig. 6 that the critical slopes might be slightly smaller on focal stimulation of one node than on bipolar stimulation on nerve trunk. The means differed by 15—30 rheobases/sec in the three types of nerve fibres. Any injury to the nerves is known to increase the rate of accommodation and injury during dissection could therefore not be the explanation of these differences. It seems therefore justified to assume that they were largely due to different stimulation techniques. The two sets of figures were combined for statistical treatment by shifting the critical slopes obtained by focal stimulation of one node 21 rheobases/sec to the right. Twentyone rheobases/sec was the mean of the difference between the means from the two types of experiments.

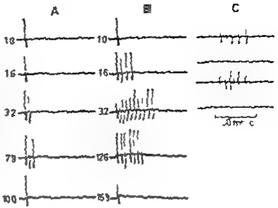

Fig 7 Discharge in a single nerve fibre innervating a fast adapting skin mechanoreceptor Nerve stimulated with long step currents at a *desheathed site* (A) and through intact nerve sheath (B) Sense organ stimulated with long mechanical pulses (C) Figures on the left hand side of the records give the intensities of the electrical pulses in units of rheobases Lower traces in (C) indicate mechanical pulse Lower amplification in (C) One action potential in (C) did not propagate to both electrodes and was therefore recorded as monophasic Retouched in (A) and (B)

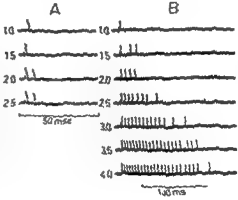

Fig 8 Repetitive activity in a single nerve fibre when stimulated with long step currents at a desheathed site (A) and through intact nerve sheath (B) Figures on the left hand side of the records give the intensities of the stimulating pulses in units of rheobases Sensory nerve fibre innervating a slowly adapting muscle mechanoreceptor For a further increase in stimulating current the number of impulses decreased in (A) as well as in (B)

The mean of the critical slopes was highest in motor fibres (113 9 ± 43 9 mean and S D) intermediate in fibres from slowly adapting muscle mechanoreceptors (85 2 ± 23 3) and lowest in fibres from fast adapting skin mechanoreceptors (66 9 ± 31 0) The results were tested for significant differences by application of the *t* test The difference was significant between motor fibres on one hand and the two types of sensory fibres on the other either taken together or separately although just barely between motor fibres and sensory fibres innervating slowly adapting receptors The difference between the sensory nerve fibres innervating the two types of mechanoreceptors was not statistically significant and the difference between the means was in the reverse direction to what would be expected if the accommodation of the nerve fibre generally was significant for the adaption of the sense organ The probability levels are shown in Table I

The critical slope varied by a factor of two to four within each group of nerve fibres except one (bottom group in Fig 6) There was no particular reason to

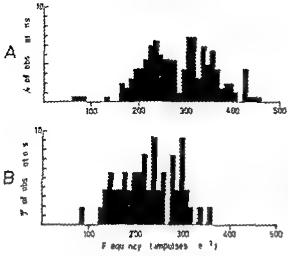

Fig 9 Histograms showing the distribution of mean nerve impulse frequencies during repetitive activity in single nerve fibres when stimulated with long step currents of varying intensities Fibres innervating fast adapting skin mechanoreceptors (A) and fibres innervating slowly adapting muscle mechanoreceptors (B)

ascribe the pronounced variation in this group to experimental error and considering that the values of the standard deviations were generally rather high it seems likely that the few low values for the critical slope in this group were extremes in the population A variation of the same order of magnitude as shown within the groups in Fig 8 has earlier been found among toad motor fibres (Sato, Nadao Terauchi Yamanaka and Matsumoto 1950 Tasaki 1950 Tasaki and Sakaguchi 1950 Sato 1951 *a*) and fibres innervating Pacinian corpuscles in the cat (Gray and Matthews 1951)

Repetitive discharge in response to electrical stimulation of single nerve fibres

Rectangular current pulses of long duration and varying intensities were applied to the nerve fibres in order to study repetition Only sensory fibres were systematically investigated in this respect Repetitive activity is illustrated in Fig 7 A and B and Fig 6 A and B It consisted of a burst of impulses with the following characteristics regardless of the experimental situation and the type of nerve fibre The discharge frequency was initially high 300—500 impulses/sec and declined slightly towards the end of the burst in most cases Occasionally the last spike interval was considerably longer than the preceding ones but a low discharge frequency for any length of time was never seen The mean impulse frequencies during single bursts are shown in histogram in Fig 9 for fibres innervating fast adapting skin mechanoreceptors (A) and slowly adapting muscle mechanoreceptors (B) presented as described above (p 6) Histogram (A) is based upon 205 observations from 11 nerve fibres which innervated 11 of the receptors investigated for Fig 2 Histogram (B) is based upon 51 observations from 9 nerve fibres Eight of these nerve fibres innervated 8 of the receptors investigated for Fig 1 The intensity of the stimulating pulse was varied over wide ranges and it seems therefore justified to state that the diagrams reasonably well

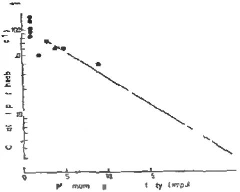

Fig 10 Critical slopes in rheobases/sec (ordinate logarithmic scale) against maximum number of impulses in the nerve fibres (abscissa) due to stimulation with long step currents Figures on the right hand side of the symbols give the number of observations represented by the symbol in case this was more than one The regression line represents the equation N = 32 6 − 15 8 log ▪ (see text p 12)

cover the maximum frequency range of these nerve fibres when stimulated with rectangular current pulses The mean impulse frequency varied roughly between 100 and 400 impulses/sec and it could not be changed by a factor of more than about two with the intensity of the stimulating pulse (Fig 7 A and B Fig 8 A and B) It is also seen in Fig 9 that the frequencies of the two types of nerve fibres were just slightly different The number of impulses increased with the intensity of the stimulating pulse up to a maximum A further increase of the pulse then caused a marked decrease in repetitive firing (Fig 7 A and B) Delayed repetition (Erlanger and Blair 1936 Sato 1951 *b*) was not seen in these experiments The maximum number of impulses from single nerve fibres varied between 1 and 24 (Fig 11) and the maximum duration of repetitive activity ever seen in a single nerve fibre was 128 msec (Fig 8 B) Repetitive firing was possible more pronounced in nerve fibres innervating fast adapting receptors than in those innervating slowly adapting receptors but the difference was rather small (Fig 11)

Relation between critical slope and maximum repetitive activity of single nerve fibres in response to electrical step stimulation

It has earlier been shown that repetitive activity to constant currents is more pronounced the slower the accommodation (Katz 1936 Sato 1951 *b*) This was confirmed in the present investigation as is seen in Fig 10 in which the critical slopes (ordinate) are plotted on a logarithmic scale against the maximum number of impulses (abscissa) which could be elicited from the single nerve fibres by stimulation with long step currents The measurements were made on 18 fibres stimulated with bipolar electrodes on the nerve trunk at one or several sites Both motor and sensory nerve fibres are represented regardless of whether they were stimulated through intact nerve sheath or at desheathed sites of the nerve It can be seen in Fig 10 that only fibres with a critical slope of less than 100 rheobases/sec discharged repetitively in response to step currents This seems to be in agreement with Sato s results (Sato 1951 *b*) since he has found that a value of

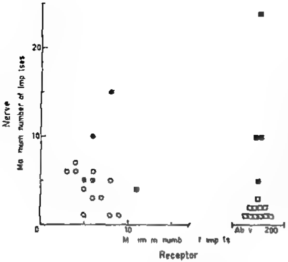

Fig 11 Maximum number of nerve impulses in single fibres when stimulated with long step currents (ordinate) against maximum number of impulses on stimulation of the attached receptors with long mechanical pulses (abscissa) Skin receptor units are indicated by circles and muscle receptor units by squares Open symbols refer to electrical stimulation at desheathed sites of the nerve and filled symbols to stimulation through intact nerve sheath

l between 10 and 30 msec is required for repetitive activity in toad motor fibres The correlation coefficient between the logarithm of the critical slope and the maximum number of impulses was highly significant ($r = -0.86$ *t* test $P < 0.001$)

The results presented in Fig 10 suggest a straight line relation between the logarithm of the critical slope and the maximum number of impulses Too few points were available from very slowly accommodating fibres however to allow a definite statement in this respect The regression line was calculated by the method of least squares using only the figures from fibres which gave more than one impulse It represents the equation

$$N = 32.6 - 15.8 \log S$$

where N is the maximum number of impulse elicited by electrical step stimulation of the nerve fibres and S is the numerical value of the critical slope given in rheobases/sec

The results obtained by focal stimulation of one node differed from those presented in Fig 10 in that fibres having a critical slope of more than about 70 rheobases/sec gave only one impulse This was a further support for the conclusion that the difference in critical slope obtained by the two types of stimulation was largely due to different experimental techniques

Maximum number of nerve impulses elicited by mechanical stimulation of receptors and electrical stimulation of their axons

The relation of maximum number of nerve impulses elicited by mechanical stimulation of receptors to that elicited by electrical step stimulation of the corresponding nerve fibres is shown in Fig 11 Fast adapting sensory units (skin receptors) are indicated by circles and slowly adapting sensory units (muscle re

ceptors) by squares In the case of the fast adapting sensory units the number of impulses was of the same order of magnitude whether produced by stimulation of the nerve fibre or of the receptor, although there was a considerable scatter On the basis of the correlation between repetitive activity and accommodation shown above (p 12) these findings seem to be in agreement with the results of Gray and Malcolm (1951) and Gray and Matthews (1951) that the critical slope of fast adapting mechanoreceptors to mechanical stimulation is of the same order of magnitude as the critical slope of their axons

The maximum number of impulses in the nerve fibres innervating the muscle receptors was on the other hand considerably smaller on electrical stimulation of the nerve than on mechanical stimulation of the sense organ the difference being ten times or more

Impulse frequency of the discharge elicited by mechanical stimulation of receptors and electrical stimulation of nerve fibres

The range of impulse frequencies obtained by electrical stimulation of single sensory nerve fibres is shown in Fig 9 The impulse frequency during repetitive activity was in only 4 observations out of 259 less than 100 impulses/sec All the slowly adapting receptors gave a maintained discharge of frequencies below 50 impulses/sec (Fig 4) Hence it is obvious that the impulse frequency range of the nerve fibres when stimulated with step currents did not cover the range of the slowly adapting receptors For the fast adapting sense organs however there seemed to be a reasonable agreement between impulse frequency range of nerve fibres and receptors (Fig 2 and Fig 9 A)

Discussion

It has been shown that accommodation and repetitive firing of nerve fibres might be affected by a number of factors such as ionic environment injury interference with circulation intact or removed connective tissue sheath temperature and in case of amphibians also temperature of the animals before the experiments (e g Erlanger and Blair 1936 Katz 1936 Parrack 1940 Tasaki 1949) Hence it is not easy to be certain that any measurement of it is normal (Gray and Matthews 1951) However to reveal differences in accommodation between nerve fibres it seems adequate to measure accommodation under standardized conditions even if these conditions are in some respect unphysiological

In the present investigation the accommodation of single nerve fibres innervating different types of end organs was measured The ionic environment was kept constant circulation was completely cut off previous and actual temperature was almost constant and the nerves were desheathed In some experiments stimulation was applied at sites where the single nerve fibres had been dissected whereas it was applied at undissected sites in others There was a slight difference in rate of accommodation obtained in these two types of experiments It seemed

very unlikely that the single nerve fibres had been subjected to injury at points where they had not been dissected while it could not *a priori* be excluded that they had been damaged at the dissection However the difference in accommodation was in the reverse direction to what would be expected if the single nerve fibres had been damaged at the dissection and the effect was probably due to the difference in stimulation technique (see Methods)

A significant difference in accommodation was found between motor and sensory nerve fibres the motor fibres being more rapidly accommodating thus confirming Erlanger and Blair (1938) and Skoglund (1942) The critical slope of motor and sensory nerve fibres did not fall in distinct groups as found by Skoglund (1942) for mammalian nerve fibres A strict single fibre technique is more suitable to determine variation between individual fibres than recording from nerve fibre bundles which was the technique used by Skoglund The difference between the results could be due either to this difference in experimental technique or to difference between the two species

A significant difference was not found between fibres innervating fast adapting skin mechanoreceptors and those innervating slowly adapting muscle mechanoreceptors Hence determination of accommodation in these nerve fibres did not give any indication of the mechanism underlying the difference in adaption between the two types of sense organs

The repetitive firing elicited by electrical stimulation of single nerve fibres was compared with the discharge due to mechanical stimulation of the attached receptors Both receptors and nerve fibres were studied under the same conditions except possibly in one respect There might have been some difference between the ionic composition of the Ringer s solution and that of the extracellular fluid at the site of impulse initiation at the receptor It seems justified to assume that the ionic environment was the same at this site as inside the nerve sheath Electrical stimulation of the nerve through intact sheath also gave repetitive activity of longer duration than stimulation at desheathed sites However this difference was not too pronounced and it seems justified to neglect it in the following reasoning concerning the relation between the discharge elicited by electrical stimulation of the nerve fibres and mechanical stimulation of the receptors

The maximum number of impulses produced by step current stimulation of the nerve fibres which innervated slowly adapting receptors was about a tenth or less than the number of impulses elicited by mechanical stimulation of the receptors If the simple generator current hypothesis for impulse initiation is correct then it must be concluded that the rate of accommodation was considerably faster in the peripheral nerve fibres than at the site of impulse initiation Low impulse frequencies which were regularly seen when these receptors were activated by extension could not be obtained from any of the nerve fibres when they were stimulated by rectangular currents The step between generator current and propagated action potentials can therefore not be explained on the

basis of the properties of the nerve fibres without further assumptions Studies on single frog muscle spindles have shown that the course of events at impulse initiation might be rather complicated and not like the situation when current is applied to a peripheral nerve fibre (Katz 1950) The finding that the frequency range of sensory discharge could not be imitated by stimulation of the peripheral nerve fibres is therefore not necessarily inconsistent with the generator current hypothesis

In the fast adapting sensory units on the other hand about the same number of impulses was obtained by stimulation of nerve fibres and receptors The impulse frequency range was also about the same Similar results were obtained by Gray and Malcolm (1951) and Gray and Matthews (1951) for other fast adapting mechanoreceptors This is consistent with the idea that the nerve membrane has the same accommodation in the peripheral nerve as at the site of impulse initiation in the receptor Loewenstein (1956) has shown however that skin mechanoreceptors which are commonly considered fast adapting may give a discharge of much longer duration when the skin is stretched than when the receptors are stimulated by maintained pressure Lower frequencies were also seen with this stimulation If this is valid for the skin receptors studied in the present investigation the stimulation technique used here might have been inadequate to elicit the maximum discharge from these sense organs and hence the agreement between receptors and nerve fibres found here might not be worth stressing too much

The results of the present investigation indicate that there is some variation in accommodation between individual nerve fibres and related to that is a variation in the tendency to fire repetitively in response to long current pulses The range of impulse frequency during repetitive firing did not seem to vary much among individual nerve fibres Further accommodation of the nerve fibre does not seem to be closely related to the type of end organ and definitely not in such a way that differences in sense organ discharge characteristics could be accounted for by the variation in nerve fibre accommodation as measured in the peripheral nerve

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From the Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm 60, Sweden

Accommodation Related to Inactivation of the Sodium Permeability in Single Myelinated Nerve Fibres from Xenopus Laevis

By

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Abstract

VALLBO, Å. B. *Accommodation related to inactivation of the sodium permeability in single myelinated nerve fibres from Xenopus laevis.* Acta physiol. scand. 1964. 61. 429—444. — Linearly rising currents were applied to single myelinated nerve fibres from the clawed toad and the critical slope for excitation was measured as an index of the rate of accommodation. A voltage clamp analysis was carried out on the same fibres and the membrane properties were related to the critical slope. It was found that there was an intimate relation between the critical slope and the steady state inactivation of the sodium permeability at potentials relative to threshold potential. The main variation in the inactivation was in the rate constant k_h. Leak conductance and sodium and potassium permeability constants did not vary closely with the critical slope. The turning on of the potassium permeability was not investigated. It was concluded that the variation in the rate constant k_h was one of the main factors accounting for the normal variation in accommodation among these nerve fibres.

Accommodation in single myelinated nerve fibres varies to some extent from fibre to fibre (e.g. Tasaki 1950, Sato 1951, Vallbo 1964). The basis for such variations seems to be unknown. The voltage clamp analyses of the squid fibre (Hodgkin and Huxley 1952 *a—d*, Hodgkin, Huxley and Katz 1952) and of the myelinated fibre (Dodge and Frankenhaeuser 1959, Frankenhaeuser 1959, 1960, 1962 *a—c*, 1963 *a*, *b*) indicate that a further analysis might reveal how fibres with different accommodation differ from each other with respect to their membrane properties. When the equations which describe the ionic currents are considered it is evident that a change of almost any of the constants, the rate constants (α and β) as well as the permeability constants (P_{Na}, P_K and P_p) would cause a change of the accommodation in the nerve model. The previous analysis indicates that the rate constants α_h, α and β vary more than the

other rate constants from fibre to fibre (Frankenhaeuser 1960, 1963 *a*) In the present investigation the accommodation of single myelinated nerve fibres was measured and a voltage clamp analysis was carried out on the same fibres The aim of the investigation was to reveal some of the mechanisms for the normal variation in accommodation Accommodation was measured with linearly increasing currents as the critical slope for excitation and the voltage clamp analysis was carried out with the method developed in this laboratory (Frankenhaeuser and Persson 1957, Dodge and Frankenhaeuser 1958)

It will be shown that there was a close dependence between accommodation, as measured by the critical slope and the steady state inactivation the slowly accommodating fibres were less inactivated at corresponding potentials relative to threshold potential The main variations of the inactivation were in the rate constant α_h *A significant correlation was not obtained between critical slope* and leak conductance (g_l), nor between critical slope and sodium or potassium permeability constants ($\bar{P}_{Na}$ and P_K)

Methods

The experiments were done on large (25—30 μ) myelinated nerve fibres from the sciatic or the peroneal nerve of *Xenopus laevis* The method used for recording membrane potential changes has been described by Frankenhaeuser (1957 *b*) and the voltage clamp technique has been described by Dodge and Frankenhaeuser (1958) The solution in the end pools of the recording cell was isotonic KCH_3SO_4 (Hopkin and Williams Essex England) A value for $A_N Z_{ED}$ of 22 5 Ω cm² was used for the current calibration (Dodge and Frankenhaeuser 1959) The Ringer s solution had the following composition (mM) NaCl 112 0 KCl 2 5 $CaCl_2$ 2 0 $NaHCO_3$ 2 5

Procedure The threshold for an action potential and the critical slope of the node were determined in the beginning and at the end of the experiment and the mean of the figures obtained from these two measurements will be presented here The rheobase was defined as the minimum current density giving rise to an action potential when the node was stimulated by a rectangular current pulse of long duration The threshold potential (V_t) was defined as the potential difference between resting potential ($V = 0$) and the potential at which the rate of change of membrane potential due to regeneration was 80 volts/sec when the node was stimulated with a long rectangular current pulse The intensity of the stimulating pulse was adjusted so that the regenerative activity appeared after about one msec The critical slope was defined as the minimum current slope which gave rise to a clear action potential (see p 5) The slope of the current was changed in steps of 10—20 %

The following voltage clamp runs were taken in each experiment (a) The pulse amplitude was varied The sodium equilibrium potential (V_{Na}) the potassium permeability constant (P_K) and the curve relating the peak sodium current to membrane potential ($I_{Na} - V$ curve) were calculated from these runs The position of this curve along the voltage axis was measured by the potential at which the peak sodium current was half maximum This potential was called V (b) Anodal pulses were applied in order to determine capacitive and leak current (c) The membrane potential was changed in two steps A long conditioning pulse (40 msec) polarized the membrane to different potentials and a test pulse to a potential ($V = 60$ mV) where the peak sodium current was about maximum The relation between steady state inactivation and the membrane

Table I Excitability critical slope and sodium equilibrium potential

Axon no.	Threshold potential, V_t (mV)	Rheobase (mA/cm)	Critical slope (mA/cm /sec)	Critical slope (rheobases/sec)	V_N (mV)
1	33.3	0.13	4.5	35	151
2	32.1	0.25	5.6	22	136
3	28.7	0.21	10.7	53	127
4	28.9	0.17	11.3	67	130
5	31.0	0.21	15.4	74	141
6	30.8	0.25	17.2	68	130
7	25.9	0.16	17.9	111	123
8	23.2	0.15	28.4	190	119
9	27.3	0.39	30.8	79	121
10	28.5	0.18	38.1	211	118
11	25.8	0.20	52.0	256	120
12	25.9	0.30	75.7	256	126
Mean	28.5	0.22			128.5
SD	3.0	0.07			10.0

potential ($h_\infty - V$ curve) was calculated from these runs The sodium permeability constant $\bar{P}_{Na}$ was calculated from (a) and (c) (d) The membrane potential was changed in two steps Amplitude and duration of the conditioning first step were varied, the amplitude between $V = -60$ and 30 mV A test step to $V = 60$ mV was applied at the end of the conditioning step The time course of inactivation was determined from these runs The voltage clamp runs (a) (b) and (c) were taken before as well as after (d) and the means of the figures obtained from the two runs will be reported here except for the permeability constants ($\bar{P}_{Na}$ and $\bar{P}_K$) which were calculated from the first sets of records.

The temperature of the preparations was kept between 19 5 C and 20 5 C during the experiments

The axons were numbered according to rate of accommodation and hence the chronological order of the experiments is neglected.

Nomenclature The nomenclature and the abbreviations were the same as those used by Frankenhaeuser and Huxley (1964) Potentials are given as inside potential minus outside potential and outward current is given as positive E is used for absolute values of the potentials V is used for potentials relative to resting potential (E_r) thus $V = E - E_r$

Formal treatment and assumptions The formal treatment and the assumptions upon which this treatment was based were the same as those used by Frankenhaeuser (1960 1963 *a*) *These in turn are slight modifications of those used by Hodgkin and Huxley (1952 a—d)* and Hodgkin Huxley and Katz (1952)

Results

Excitability

The threshold for an action potential was determined The results from these measurements are shown in Table I The threshold potential (V_t) was 28 5 ± ± 3 0 mV (mean and S D) The threshold potential depends to a great extent

other rate constants from fibre to fibre (Frankenhaeuser 1960 1963 a) In the present investigation the accommodation of single myelinated nerve fibres was measured and a voltage clamp analysis was carried out on the same fibres The aim of the investigation was to reveal some of the mechanisms for the normal variation in accommodation Accommodation was measured with linearly increasing currents as the critical slope for excitation and the voltage clamp analysis was carried out with the method developed in this laboratory (Frankenhaeuser and Persson 1957 Dodge and Frankenhaeuser 1958)

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6	30.8	0.25	17.2	[illegible]	130
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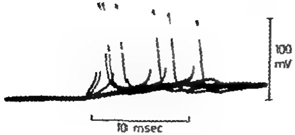

Fig 1 Membrane potential records from a single node when stimulated by linearly rising currents of various slopes Seven superimposed records

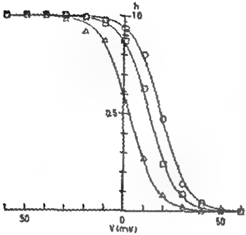

Fig 2 Steady state inactivation (ordinate) plotted against membrane potential (abscissa) Triangles from axon 11 squares from axon 5 and circles from axon 2 Continuous lines calculated from eqn (1)

upon the rate constants α_m and β_m according to the voltage clamp treatment (Frankenhaeuser 1960) In order to check if the variation in threshold potential found in the present investigation was attributable to a variation in these two rate constants the position of the $I_{N} - V$ curve relative to threshold potential was determined The correlation between V i e the potential at which the peak sodium current was half maximum and V was highly significant ($r = 0.76$ t test $P < 0.001$) The regression coefficient of V_t on V was 0 56 It seems justified to conclude from this that the variation in threshold potential was largely accounted for by a variation in the position of the $I_{N} - V$ curve along the voltage axis and hence by a variation in the constants α_m and β_m The fact that the regression coefficient was below one seems to indicate that a shift of $I_{N} - V$ curve by a certain amount might give a smaller shift in threshold potential or/and that variations in other factors counteracted the effect on threshold potential of the shift in $I_{N} - V$ curve The rheobase was 0.22 ± 0.07 mA/cm² (mean and S D) A significant correlation was not present between rheobase and threshold potential (t test P 0 8)

The sodium equilibrium potential (V_N) was 128.5 ± 10.0 mV (mean and S D) About the same mean value for sodium equilibrium potential (126 3 and 121 0 mV) has been found by Dodge and Frankenhaeuser (1959) and Frankenhaeuser (1959)

Table II Steady state inactivation

Axon no	V_h (mV)	k_h (mV)	h_∞
1	20 0	7 5	0 94
2	18 8	7 0	0 94
3	10 5	6 5	0 83
4	14 0	7 5	0 89
5	13 0	7 0	0 87
6	13 5	6 0	0 91
7	8 0	7 0	0 80
8	2 9	7 0	0 60
9	8 3	7 5	0 75
10	5 2	6 5	0 71
11	2 8	7 0	0 62
12	4 0	7 0	0 67

Accommodation

Examples of membrane potential changes in response to linearly rising currents of varying slopes are shown in Fig 1 Seven records were superimposed In six of them the linearly increasing current elicited an action potential and in one only a small hump and a delayed rectification The slope of the stimulating current was highest in the record with the first action potential and the following action potentials were elicited by currents of successively smaller slopes Regeneration started at about the same potential regardless of the slope of the stimulating current Hence the change in threshold potential was minute (cf Tasaki 1950, Frankenhaeuser 1952) The action potential decreased smoothly with the slope of the stimulating current until there was a sudden failure of regeneration Essentially the same results have been reported by Frankenhaeuser (1952) Very much smaller action potentials than the last one recorded in Fig 1 were usually not seen in response to linearly rising currents and it seems therefore that the curve relating the response of the membrane to the slope of the stimulating current was very steep in this region The critical slope was defined above as the minimum current slope which elicited a clear action potential The records in Fig 1 show that there was no problem in practice to decide whether the stimulating current gave rise to an action potential or not in spite of the fact that the size of the action potential varied with the slope of the stimulating current The critical slopes are given in Table I in two units first as rate of change of membrane current density (mA/cm²/sec) and secondly as rate of change of membrane current in units of rheobasic strength (rheobases/sec) There was a fairly good straight line relation between these two sets of figures although some scatter was present This scatter might be due to a variation in internodal length and nodal membrane area among the fibres These variations were not accounted for in the calculations since a standard value for $A_N Z_{ED}$ was used for the current calibration

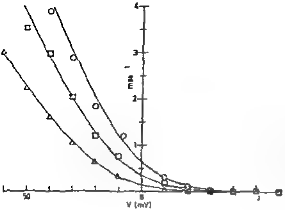

Fig 3 Rate constant α_h (ordinate) plotted against membrane potential (abscissa) Axon 11 (triangles) axon 5 (squares) and axon [illegible] (circles) Same axons as in Fig 2 Continuous lines calculated from eqn (2)

Inactivation of the sodium permeability

An empirical equation was fitted to the experimental values for the steady state inactivation (Frankenhaeuser 1959)

$$h_\infty = \frac{1}{1+\exp\left[(V-V_h)/k_h\right]} \tag{1}$$

This equation [illegible] an approximation for the equation (9) given by Frankenhaeuser (1960) Figure 2 shows the $h_\infty - V$ curve for three different fibres The numerical values for V_h and the constant k_h as well as the steady state inactivation [illegible] resting potential h_0 are given in Table II The $h_\infty - V$ curve had nearly the same shape in all the fibres as is evident from the rather small variation in the constant k_h The position of the curve along the voltage axis on the other hand varied considerably the extreme values for V_h being 2 8 and 20 0 mV About the same values for V_h k_h and h_0 have been reported earlier by Frankenhaeuser (1959) for myelinated nerve fibres from the same species with the exception that a slightly higher variation in k_h has been found by Frankenhaeuser

The rate constant α_h was calculated and an empirical equation of the following form was fitted to the experimental data from each fibre (Frankenhaeuser 1960 eqn 18)

$$\alpha_h = A(B-V)/\left[1-\exp\left(\frac{V-B}{C}\right)\right] \tag{2}$$

The rate constant α_h of the three fibres from Fig 2 is shown in Fig 3 These plots illustrate the general finding that α_h was higher at corresponding potentials the higher the value of V_h The values of the constants A B and C in the empirical equation fitted to the experimental values for α_h are given in Table III There was a considerable variation in both A and B indicating that the shape of the curve as well as the position along the voltage axis varied among the fibres

Table III Rate constant α_h leak conductance and sodium and potassium permeability constants

Axon no	Constants in eqn (2) A	B (mV)	C (mV)	g_L (m mho/cm)	P_{Na} (cm/sec × 10^{-3})	P_K (cm/sec × 10^{-3})
1	0.13	−13	9	9.5	18.1	1.25
[illegible]	0.12	− 6	8	23.0	10.5	0.97
3	0.11	−16	8	20.0	4.8	0.80
4	0.13	−15	8	16.0	5.4	0.68
5	0.10	−12	8	17.0	9.2	0.84
6	0.14	−18	9	15.0	8.3	0.81
7	0.10	−13	8	19.0	9.2	1.20
8	0.055	− 9	[illegible]	23.0	8.8	1.05
9	0.09	−12	7	32.0	6.9	1.13
[illegible]	0.07	−13	7	17.0	6.6	0.86
11	0.07	−18	8	25.0	7.3	0.84
12	0.08	−23	8	34.0	5.1	0.70
Mean				20.9	8.3	0.93
SD				7.0	3.6	0.19

The rate constant β_h was calculated between $V = -60$ and 30 mV Hence only the lower part of the curve relating the value of β_h to membrane potential was available It was therefore not possible to fit an empirical equation reasonably correctly to the values from each experiment However β_h varied only little among the fibres (see below and Fig 4) and a fairly good fit to all the experimental points was obtained by the same equation as the one given by Frankenhaeuser (1960 eqn 19) for β_h with the exception that the first right hand factor was 0 03 instead of 0 05 as in the original equation Hence β_h was slightly lower at corresponding potentials in the present investigation This difference is small and it is very likely insignificant with regard to the errors in the measurements

The potential (V_h) at which the steady state inactivation was half complete is plotted against the experimental values for α_h and β_h at $V = 10$ mV in Fig 4 The rate constant α_h (filled circles) varied by a factor of 7 7 and V_h increased with α_h The other rate constant for inactivation β_h (open circles) varied by a factor of only 2 1 and V_h decreased with β_h A corresponding relation as in Fig 4 between V_h and the relative values for α_h and β_h was observed at $V = 20$ and 30 mV A shift of the $h_\infty - V$ curve in the positive direction along the potential axis implies a higher value of α_h and/or a lower value of β_h at corresponding potentials (Frankenhaeuser 1960 eqn 9) The results presented above justify the conclusion that the variation in the position of the $h_\infty - V$ curve found in the present investigation was mainly accounted for by a variation in the rate constant α_h although a variation in β_h contributed slightly to this

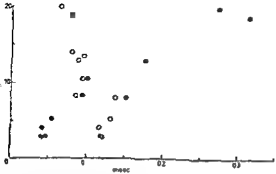

Fig 4 V_h (ordinate) plotted against rate constant α_h (filled circles) and β_h (open circles) at $V = 10$ mV (abscissa)

Leak conductance

The leak conductance (g_L) was calculated from the steady state currents during anodal pulses according to the following equation (Frankenhaeuser 1960)

$$g_L = \frac{I_l}{V - V_L} \tag{3}$$

where g_L is the leak conductance I_l the steady state current during an anodal voltage clamp pulse to the potential V and V_L is the equilibrium potential for the leak current and its value is $\simeq 0$ The leak conductance was $20\,9 \pm 7\,0$ m mho/cm² (mean and S D) The values from each nerve fibre are given in Table III A slightly higher mean value (30 3 m mho/cm²) and a smaller variation in the leak conductance have been reported by Frankenhaeuser (1960)

Sodium permeability constant

The sodium permeability at the peak of the initial current when the membrane potential was changed in steps from resting potential was calculated according to the equation used by Dodge and Frankenhaeuser (1959 eqn 2) The maximum value of this peak sodium permeability was obtained from a plot of the permeability against membrane potential The maximum value was reached well before $V = 100$ mV (cf Dodge and Frankenhaeuser 1959) at which potential the variable m is approximately equal to one in the steady state (Frankenhaeuser 1960) The values of $\bar{P}_{Na}$ for the fibres investigated were calculated from the recorded currents using the value for τ_m/τ_h at 100 mV steps earlier found by Frankenhaeuser (1960) The sodium permeability constant was $8\,3 \pm 3\,6 \times 10^{-3}$ cm/sec (mean and S D) Frankenhaeuser (1960) has reported about the same mean value ($8\,0 \times 10^{-3}$ cm/sec) The sodium permeability constant from each fibre is given in Table III

Potassium permeability constant

The potassium permeability constant ($\bar{P}_K$) was calculated from the steady state currents at large cathodal steps according to the equation used by Frankenhaeuser (1962 c eqn 1) The potassium permeability constant was $0\,93 \pm$

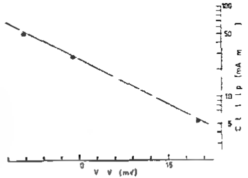

Fig 5 Critical slope (ordinate logarithmic scale) plotted against the potential difference ($V_A - V_t$) (abscissa) The straight line represents eqn (4)

0 19 × 10^{-3} cm/sec (mean and S D) This value is slightly lower than the value (1 20 × 10^{-3} cm/sec) found by Frankenhaeuser (1962 *c*) The individual figures for P_K are given in Table III

There was not a significant correlation between the two permeability constants ($\bar{P}_N$ and P_K $r = 0\ 57$ *t* test $P > 0\ 05$) and thus it is not possible to attribute the variations in the two permeability constants solely to variations in internodal length and nodal membrane area

Relation between accommodation and voltage clamp data

The three $h_\infty - V$ curves shown in Fig 2 were obtained from axons having widely different accommodation Axon 11 (left hand curve triangles) had about ten times higher critical slope than axon 2 (right hand curve circles) and axon 5 (squares) had a critical slope inbetween these two The figure illustrates the general finding that the smaller the critical slope the more the $h_\infty - V$ curve was shifted in positive direction (see Table II) However for a discussion of inactivation of the sodium permeability as related to accommodation it seems more appropriate to consider the position of the $h_\infty - V$ curve along the voltage axis relative to the threshold potential than to the resting potential (see Discussion p 14) This relation was approximated as the potential difference ($V_A - V_t$) Figure 5 is a plot of the critical slope in mA/cm²/sec (logarithmic scale) against ($V_A - V_t$) (linear scale) It is seen that the smaller this potential difference the lower was the critical slope A greater part of the sodium permeability was thus available in the steady state at any potential relative to threshold potential in fibres with slow accommodation than in fibres with fast accommodation Figure 5 also suggests a straight line relation between the logarithm of the critical slope and ($V_A - V_t$) The correlation between these two quantities was highly significant (*t* test $P < 0\ 001$) The correlation between the logarithm of the critical slope in units of rheobases/sec and ($V_A - V_t$) was of the same significance (see Table IV) The regression line in Fig 5 which was calculated with the method of least squares represents the equation

$$Y = -0\ 1\ X - 0\ 59 \qquad (4)$$

Table IV Correlations between critical slope (log critical slope) and voltage clamp data

	Critical slope in units of mA/cm²/sec			Critical slope in units of rheobases/sec		
	r	P		r	P	
$(V_h - V_t)$ and log critical slope	−0 93	0 001>P	Highly significant	−0 93	0 001>P	Highly significant
$\bar{P}_{Na}$ and critical slope	−0 46	0 3 >P>0 2	Insignificant	−0 38	0 3>P>0 2	Insignificant
$\bar{P}_K$ and critical slope	−0 32	0 4 >P>0 3	Insignificant	−0 32	0 4>P>0 3	Insignificant
g_L and critical slope	0 71	0 01>P>0 001	Significant	0 49	0 2>P>0 1	Insignificant

r correlation coefficient P probability

where Y is the logarithm of the numerical value for the critical slope in mA/cm²/sec and X is the numerical value for $(V_h - V_t)$ in mV

The correlation coefficients between the critical slopes on one hand and the permeability constants ($\bar{P}_{Na}$ and $\bar{P}_K$) and the leak conductance (g_L) on the other are given in Table IV together with the correlation coefficients between the logarithm of the critical slope and the potential difference $(V_h - V_t)$ The significance of the correlations were tested by application of the t test to the data and the probability levels are listed in the same Table

The correlation between the leak conductance and the critical slope in mA/cm²/sec was significant but was not significant between the leak conductance and the critical slope in rheobases/sec These findings will be further discussed on p 15 Significant correlations were not obtained between the critical slope on one hand and the two permeability constants on the other These correlations were calculated by using the figures obtained from the first measurement of the critical slopes and not the means of the figures obtained in the two measurements (see Methods) The reason for this was that the permeability constants were calculated from only the first voltage clamp runs and thus these quantities were determined from records taken close in time during the experiments

Repetitive activity

Slowly accommodating fibres gave often more than one action potential in response to a stimulating step current of long duration Membrane potential records during such repetitive firing are shown in Fig 6 All but the first action potential decreased with the intensity of the stimulating pulse and so did the intervals between the action potentials It seems likely that these findings are predicted by the equations describing the ionic currents

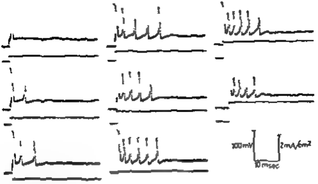

Fig 6 Repetitive firing of a node in response to step currents of various amplitudes Upper trace membrane potential Lower trace stimulating current

Discussion

In the present investigation the sodium permeability was studied in single myelinated nerve fibres The response of the same nerve fibres to linearly increasing currents was measured in order to determine the critical slope for excitation The critical slope was considered an adequate expression for the rate of accommodation of the fibre The aim of the investigation was mainly to find out if the sodium permeability varied in a relatively simple and systematic manner with the critical slope in such a way that it would be justified to conclude that the variation in the sodium permeability could be one of the factors accounting for the normal variation in accommodation It was realized that accommodation would be affected by almost any of the constants which determine the ionic currents in the nerve according to the voltage clamp treatment

The major finding was that the rate of accommodation measured as the critical slope closely and systematically depended on the difference between threshold potential and the potential at which the inactivation was half complete in the steady state

Excitability measurements

Threshold was measured in two units (a) Minimum current density required to evoke an action potential when the membrane was stimulated by a step current of long duration This figure is equivalent to the rheobase in this experimental situation (b) The potential (I) at which the rate of change of membrane potential due to regeneration was 60 volts/sec This definition gives certainly somewhat higher value for threshold potential than the minimum potential required for regeneration which potential would correspond to the theoretical threshold potential of the nerve model However the threshold potential as defined here seems adequate enough for the purpose of the present study since mainly the variations in threshold potentials among the fibres are considered

Critical slopes were also measured in two units (a) Rate of change current density (mA/cm²/sec) (b) Rate of change of current where current was measured in units of rheobasic strength (rheobases/sec) These two units are not equivalent Rheobases/sec seems to be a more appropriate figure for the rate of accommodation than mA/cm²/sec However there was a reasonably good straight line relation between the two sets of figures and in most cases a calculation of the relation between the voltage clamp data and the critical slope gave about the same results regardless of which unit for critical slope was used (see Table IV) The relation between leak conductance and critical slope was one exception which will be discussed on p 15

Before the discussion of the main findings the uncertainties in the potential and current measurements will be briefly considered The potentials are given in T[illegible] as potentials relative to resting potential according to the definition given in Methods It has been shown that the resting potential of the types of nerve fibres studied in the present investigation is about —70 mV across the membrane (Dodge and Frankenhaeuser 1959) However $V = 0$ is not more than *an approximation for the resting potential* and the absolute value of the membrane potential (E) corresponding to the potential $V = 0$ might have varied slightly from one experiment to another due to the state of the fibre and a variation in the attenuation artefact (Dodge and Frankenhaeuser 1958) It is therefore possible that some fibres were polarized by a few mV in one direction [illegible] the other during the experiments A polarization would give a shift of all the calculated potentials by the same amount from the correct values in one and the same fibre The variations found in V_A V_t and V_N were not consistent with the idea that polarization was the main mechanism for these variations Hence it seems justified to conclude that the variations in the calculated potentials were mainly physiological in spite of the uncertainties in the measurements

The figures based upon measurements of current might be scattered due to variation in the value for $A_N Z_{ID}$ The value used here for the calculations is representative for the average nerve fibres of the type studied in the present investigation (Dodge and Frankenhaeuser 1959) but some variation in internodal length and nodal membrane area was very likely present among the fibres A higher value for $A_N Z_{ID}$ than that used for the calculations would give a too high value for $\bar{P}_{Na}$ $\bar{P}_K$ leak conductance rheobase and critical slope in mA/cm² sec.

The extreme values of threshold potential (V_t) differed by as much as 10 mV This is of the same order of magnitude as found by Frankenhaeuser (1957 *a*) Several factors could account for this variation It has been shown that anodal polarization does not change the threshold potential when the calcium concentration in the surrounding fluid is the same as in the Ringer's solution used here (Frankenhaeuser 1957 *a*) This suggests that the normal variation in h_0 and n_0 was not an important factor for the normal variation in threshold potential High leak conductance and low $\bar{P}_{Na}$ would tend to increase threshold poten

tial In the present investigation high leak conductance and low $\bar{P}_{Na}$ were however more commonly associated with low than high threshold potential The position of the I_{Na} — V curve along the voltage axis varied closely with threshold potential indicating that a variation in α_m and β_m accounted for the variation in threshold potential It is possible that the variation in $\bar{P}_{Na}$ and leak conductance found here somewhat canceled the effect on threshold potential of the variation in these two rate constants The low regression coefficient between the threshold potential (V_t) and the potential (V) at which the peak sodium current was half maximum is clearly consistent with such an idea

Inactivation of the sodium mechanism

There was a pronounced variation in the inactivation of the sodium permeability among the fibres The position of the h_∞ — V curve along the voltage axis varied considerably but the curve had nearly the same shape in all the fibres A shift of the h_∞ — V curve in the positive direction implies a higher value of α_h and/or a lower value of β_h at corresponding potentials (Frankenhaeuser 1960 eqn 9) In the present investigation β_h was fairly constant while α_h varied considerably and the higher the value of this rate constant at corresponding potentials the higher was the value of V_h Hence the variation in the position of the h_∞ — V curve was mainly accounted for by a variation in α_h The curve relating the experiment values of α_h to membrane potential was generally shifted to the right it was steeper at low potentials and the bend of the curve was sharper the higher the value of V_h The variation in α_h is reflected as a variation in all the three constants (A B and C) of the equation fitted to the experimental values of α_h (see Table III) As a simple expression for the variation in the inactivation the potential V_h will be considered in the discussion concerning the relation between accommodation and inactivation Thus the small variation in the shape of the h_∞ — V curve and the variation in the time course of the inactivation will not be considered in the following discussion

Accommodation and voltage clamp findings

The changes in membrane potential in response to cathodal current pulses are associated with currents which are complex and governed by a number of factors according to the voltage clamp treatment Regenerative activity in the nerve model during a stimulating pulse requires that the system reaches a state at which the derivative of the total ionic current with respect to membrane potential (dI/dV) changes from positive to negative values This state is not defined in a simple manner by the equations describing the ionic currents It is obvious however that a decrease of the inward ionic current and an increase of the outward ionic current implies an approach towards the state at which regeneration cannot occur All the constants in the nerve model are significant for the ionic currents at any time and potential A rather extensive discussion

Critical slopes were also measured in two units (a) Rate of change current density (mA/cm^2/sec) (b) Rate of change of current where current was measured in units of rheobasic strength (rheobases/sec) These two units are not equivalent. Rheobases/sec seems to be a more appropriate figure for the rate of accommodation than mA/cm^2/sec. However, there was a reasonably good straight line relation between the two sets of figures and in most cases a calculation of the relation between the voltage clamp data and the critical slope gave about the same results regardless of which unit for critical slope was used (see Table IV) The relation between leak conductance and critical slope was one exception which will be discussed on p 15

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The extreme values of threshold potential (I) differed by as much as 10 mV This is of the same order of magnitude as found by Frankenhaeuser (1957 *a*) Several factors could account for this variation It has been shown that anodal polarization does not change the threshold potential when the calcium concentration in the surrounding fluid is the same as in the Ringer s solution used here (Frankenhaeuser 1957 *a*, This suggests that the normal variation in h_I and r_I was not an important factor for the normal variation in threshold potential High leak conductance and low $\bar{I}_K$ would tend to increase threshold poten

The outward ionic current at any time and potential is greater the higher the leak conductance. It seems therefore that a variation in leak conductance could affect the rate of accommodation. A significant correlation was not found between leak conductance and critical slope in units of rheobases/sec. This suggests that the variation in leak conductance was not a main factor for the normal variation in rate of accommodation. When a nodal membrane is stimulated with a linearly rising current the membrane potential changes with a fairly constant slope as long as the membrane is roughly equivalent to an RC-circuit, i. e. up to about threshold potential. The slope of the membrane potential change is proportional to the membrane resistance under these conditions. The specific permeabilities are dependent on membrane potential and time but current independent as found in the voltage clamp analysis. It is therefore not a surprising finding that the correlation coefficient between leak conductance and critical slope in units of mA/cm²/sec was higher than between leak conductance and critical slope in units of rheobases/sec. It is obvious from the above discussion that this finding does not warrant any further conclusions about the significance of the leak conductance for the normal variation in accommodation as measured in the more conventional way, i. e. in units of rheobases/sec. It seems very likely, however, that the variation in critical slope in units of mA/cm²/sec found in the present investigation was partly accounted for by the variations in the leak conductance since there was a significant correlation between leak conductance and critical slope measured in these units (see Table IV).

Concluding remarks

The results of the present investigation indicate that a variation in the inactivation of the sodium permeability was an important factor for the normal variation in rate of accommodation in single myelinated nerve fibres. The rate constant α_h was higher the slower the accommodation while β_h did not vary to any great extent among the fibres. No definite support was obtained for the idea that variations in the sodium permeability constant, the potassium permeability constant or the leak conductance were important factors for the normal variation in accommodation. In many cases, however, high sodium permeability constants and low leak conductances were associated with low critical slopes and *vice versa*, which finding might suggest that these factors could be of some importance although significant correlations were not found in this small number of experiments. The rate constants describing the changes in the potassium permeability were not investigated. It seems clear that a variation in this mechanism could also affect the rate of accommodation. Nor was the non specific current (I_p) (Frankenhaeuser 1963 *a*) studied. This current is rather small, however, compared to the other ionic currents and it seems therefore unlikely that a variation in this system would affect the accommodation to any great extent.

The results presented do not give a complete answer to the question concerning which factors account for the normal variation in accommodation. Nor

spleen (Celander 1954) and on the intestinal resistance vessels (Koch 1959) but not on the relationship between constrictor fibre discharge and the effector response of the consecutive sections of the intestinal vascular bed To study such aspects in more detail a series of investigations has been begun utilizing a modification of a technique developed in this department (Mellander 1960) With this technique resistance response in both the pre and postcapillary sections capacitance response response of precapillary sphincters and the net transfer of fluid across the capillary bed can be studied simultaneously In one study (Folkow Lundgren and Wallentin 1963) these functions were investigated over the whole range of intestinal vascular smooth muscle tone constriction being produced with l noradrenaline and dilatation by isopropyl noradrenaline

With this knowledge as a background the present study was undertaken in order to estimate the extent to which graded stimulation of the vasoconstrictor fibres to the intestine could influence total regional flow resistance, regional blood volume and the net transfer of fluid across the capillary membrane which provides information on the ratio of pre to postcapillary resistance In the companion paper the effect of vasoconstrictor fibre stimulation on the size of the intestinal capillary surface area available for fluid exchange will be described These two studies on the influence of graded constrictor fibre activation on the intestinal vessels were considered as necessary preliminary steps to an understanding of the splanchnic vascular responses to reflex or central nervous influences in situations in which the vasomotor fibre connections are intact Part of the present results have been reported briefly (Lewis Folkow and Mellander 1962 Folkow *et al* 1963)

Methods

Observations were carried out on a total of 60 cats fasted 24 hours After induction with ether they were anesthetized intravenously either with chloralose alone (50 to 60 mg/kg) with chloralose (50 mg/kg) combined with urethane (100 mg/kg) or with nembutal (30 mg/kg)

The technique utilized here has been described by Folkow Lundgren and Wallentin (1963) and this paper should be consulted for details The preparation is illustrated schematically in Fig 1

The greater part of the jejunum ileum (40 to 60 g of tissue was enclosed in a pl th vsmograph filled with Tyrode s solution This permitted a continuous recording of changes in intestinal volume Blood flow was measured from the cognate vein draining the intestine using a drop recorder unit operating an ordinate writer The venous outflow pressure was measured with a saline manometer and could be set at any desired level by adjusting the height of the tube end which drained the drop recorder Mean arterial blood pressure was recorded from the right femoral artery by means of a mercury manometer It was kept constant in most of the experiments by adjustment of a screw clamp placed around th aorta just proximately to the coeliac and superior mesenteric arteries Heparin was used a th anticoagulant The splanchnic nerves of both sides were cut just beneath the diaphragm and the peripheral ends mounted on stimulation electrodes In several experiments only the left side nerves were stimulated Supramaximal square wave pulses [illegible] to [illegible] V [illegible] to 3 msec were delivered to the splanchnic

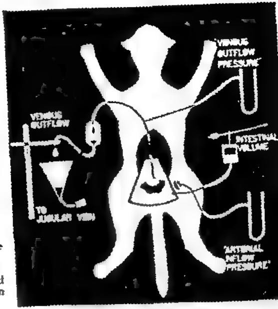

Fig. 1 Schematic illustration of the technique used for studying the reactions of pre- and postcapillary resistance vessels, capacitance vessels and transcapillary filtration exchange in the intestine

nerves from a Grass stimulator (model S4E). Frequencies between 1 and 16 imp/sec were studied with occasional observations at 32 imp/sec.

In most of the experiments secretion from the adrenal medulla was eliminated. For this purpose the right adrenal gland was isolated completely from the circulation by encircling ligatures and the left gland denervated by severing its nerve branches without interfering with its blood supply so as to preserve secretion of corticoids. If there was any question as to the blood supply to this gland the animal was given 10 mg of hydrocortisone intramuscularly.

At the start of the experiment the venous pressure usually was adjusted to bring the intestine into an isovolumetric state, which could be expected to correspond to the normal. In several experiments, however, in which it was intended to study the vasoconstrictor fibre influence on the veins distended by an increased transmural pressure, the venous outflow pressure was raised well above the isovolumetric level just before stimulation.

In most experiments the vagal nerves were cut in order to avoid the interference of centrally induced vagal influences on intestinal motility and secretion. In addition atropine was often given to supress local reflexes which might affect intestinal motility and secretion intermittently and thus disturb the steady state resting condition (cf. Porciuncula and Crowell 1963).

At the conclusion of a number of the experiments an estimation of the blood volume of the tissue within the plethysmograph was made for calculating the fraction expelled by vasoconstrictor fibre activation, using the method previously described (Folkow, Lundgren and Wallentin 1963).

Results

Fig. 2 and 3, taken from two different experiments, show typical responses of the intestinal vasculature to regional sympathetic vasoconstrictor nerve fibre stimulation. The effect of nerve stimulation on blood flow is similar in both

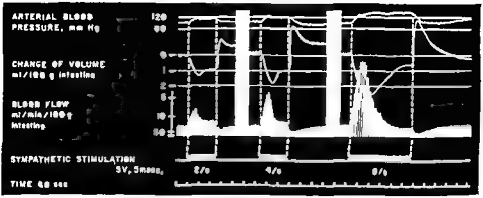

Fig 2 Cat 3 0 kg Bilateral splanchnic nerve stimulations Resistance increases rapidly upon stimulation and reaches a peak at approximately 1 to 2 min from which point it then declines At 2 and 4 imp/sec the steady state resistance level is greater than control whereas at 8 imp/sec it is *less* With cessation of stimulation there is a reactive hyperemia Volume declines rapidly upon stimulation (active and passive blood expulsion) and is to some extent reversed (mainly passive-elastic component) with the declining resistance following which a new steady state of volume is reached With cessation of stimulation there is an overshoot in regional blood volume Venous outflow pressure maintained at approximately 3 mm Hg

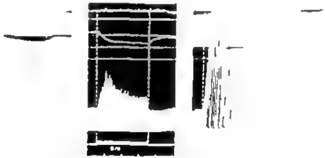

Fig 3 Cat 2 0 kg Similar experiment as that shown in Fig 2 with the exception that arterial inflow pressure was kept constant during stimulation and venous outflow pressure was at a slightly higher level The pattern of the resistance response is similar to that in Fig 2 but the change of volume is different in that virtually only the active expulsion of blood from the region is demonstrated By maintaining a slightly elevated venous outflow pressure the passive elastic recoil of the veins was minimized During steady state constriction the tissue is nearly isovolumetric indicating no net transfer of fluid across the capillary

figures but the change of volume is different due to the fact that the venous outflow pressure was higher in Fig 3

To facilitate the description of the results the resistance responses will be dealt with in detail first This will make the analysis of the capacitance responses easier to describe since the problem of the passive-elastic recoil of the veins

must be considered whenever there is a drop in transmural pressure within the venous compartment due for example to a neurogenic vasoconstrictor influence on the resistance vessels The veins being highly distensible will exhibit a passive elastic recoil The extent of this passive-elastic recoil will depend in part on the initial venous outflow pressure as will be described in more detail below This change in capacitance must be differentiated from that caused by a true neurogenic constriction of the capacitance vessels themselves

The neurogenic constriction of the intestinal resistance vessels characteristically consisted of two phases There was first a brief but often intense peak response, reaching its maximum within 30 to 40 sec This subsided within 2 to 3 min a phenomenon which will be called autoregulatory escape from the constrictor fibre influence for reasons discussed below This first phase then went over into a second phase of less pronounced but generally well maintained constriction When the stimulation was ended a reactive hyperaemia followed

The peak response increased markedly with increasing rate of stimulation but so did the extent of the autoregulatory escape The magnitude of some of the peak responses obtained at frequencies of 8 imp/sec or higher suggested a shortlasting critical closure in all probability located in the precapillary resistance section However for steady state neurogenic control of the vessels the extent of the second phase of constriction must be more relevant The steady state resistance response was of the order of a 20 to 30 per cent increase above the control level at a frequency of 1 imp/sec The maximal steady state response only exceptionally more than 50 to 100 per cent increase was obtained at 4 to 6 imp/sec At higher rates the increase in resistance in the steady state phase was on the average less than at 4 to 6 imp/sec and on occasion flow resistance after the initial peak response became less than control Even in these cases there was a reactive hyperaemia following cessation of stimulation (see e g the effect of stimulation at 8 imp/sec in Fig 2) which suggests a redistribution of blood flow within the intestine during stimulation since nerve fibre activation did not reduce total flow (*cf* Folkow *et al* 1964)

In addition to total resistance response the technique used here permits analysis also of the response of both the pre and postcapillary resistance vessels With constant arterial and venous pressures an increase in the ratio of pre to postcapillary resistance would cause a decrease in mean capillary pressure resulting in a net movement of fluid from the extravascular space into the circulation which in turn causes a decrease in the plethysmographic volume recording Such a train of events is seen when skeletal muscle vasoconstrictor fibres are stimulated (Mellander 1960) On the other hand a decreased ratio would produce outward filtration The present study revealed that in the intestine there seems to be no significant shift in this ratio during the steady state phase of constrictor fibre stimulation Evidently nerve stimulation not only increases both pre and postcapillary resistances but these effects are so balanced during the steady state response that mean capillary pressure is not changed

There was nonetheless fluid in the extravascular space of the intestine which could move into the circulation for, if during the steady state response one decreased venous pressure a procedure known to decrease mean capillary pres sure there was a steady decline in tissue volume In more than 300 stimula tions evidence of a slight inward filtration has been observed in only 4 to 5 cases and at best the decrease of mean capillary pressure in these exceptional cases was estimated as being no more than 1 to 2 mm Hg However during the initial peak response there seemed to be a considerable but very shortlasting increase in the pre to postcapillary resistance ratio causing an often marked lowering of transmural pressure in the capillary and venous compartments This is obvious from the often pronounced passive elastic recoil of the venous capacitance compartment which can be seen from the marked but short lasting tissue volume decrease particularly pronounced when venous outflow pressure is low favouring collapse of these vessels (Fig 2) Even if arterial blood pressure increased during nerve stimulation the tissue remained isovolumetric during the steady state response which must mean that the pre to postcapil lary resistance ratio increased just enough to maintain a constant mean capil lary pressure On occasion during prolonged stimulations at higher rates where the autoregulatory escape was especially pronounced, there was a slow out ward filtration In other words the neurogenic postcapillary resistance response appeared to be relatively better maintained in the long run so as to occasional decrease the pre to postcapillary resistance ratio a phenomenon observed in skeletal muscles only when their vessels have been exposed to a prolonged and severe ischaemia (Lewis and Mellander 1962)

In this connection it should be stressed that the normally existing flow re sistance of the portal vascular bed of the liver was bypassed in the present ex periments This vascular bed normally makes up part of the postcapillary re sistance for the intestinal vascular bed and its flow resistance may be expected to increase to some extent during a generalized increase of sympathetic dis charge If anything therefore there might be a still greater possibility in the intact organism to reach a situation of a slow outward filtration across the in testinal capillary walls in states of intense and prolonged sympathetic activity due to a decrease in the pre to postcapillary resistance ratio In a few experi ments attempts were made to measure roughly the extent to which portal flow resistance might increase when the hepatic vessels were exposed to the same constrictor fibre discharge as the intestinal vessels The venous blood emerging from the drop recorder was then led into the proximal end of the portal vein and it was observed to what extent the inflow rate into the liver vessels decreased for a given pressure head during activation of the constrictor fibres to the hepatic vessels Approximate evaluations indicated that the slight in crease of intrahepatic resistance that could be observed would have raised the capillary pressure in the intestine some 1 to 2 mm Hg if connected directly in series with the intestinal vessels This is no doubt a small figure but potentially

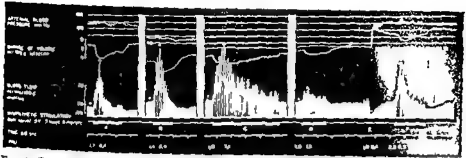

Fig 4 Cat 3 2 kg Effects of sympathetic vasoconstrictor fibre stimulation on the intestinal vasculature at various levels of arterial perfusion pressure
A. Responses during normal blood pressure (control)
B Responses after a 50 min period in which blood pressure was kept at 55 mm Hg by partial aortic occlusion
C Responses after a 5 min period in which blood pressure was kept at 30 mm Hg (No restoration of blood pressure between B and C)
D Responses after a 28 min period in which blood pressure was kept at 30 mm Hg
E. Restoration of blood pressure
F Repeated stimulation at control blood pressure The dots indicate the points in which peripheral resistance (PRU) was calculated

it may be highly important considering the big capillary surface area in the intestine (Folkow Lundgren and Wallentin 1963) Further experiments primarily directed towards such pathophysiological consequences of intense and prolonged vasomotor discharge are however needed in order to analyse such disturbances in more detail

In some experiments arterial pressure was kept lowered at a constant level for up to 30 to 60 min usually first at 60 mm Hg then at 35 to 40 mm Hg Due to the pronounced autoregulation of the precapillary resistance vessels flow was only moderately lowered by the pressure drop until a pressure level of some 35 mm Hg was reached where evidently the vessels generally reached the maximally dilated state At further pressure drops flow decreased profoundly and resistance could not be lowered any more As long as the vessel still showed autoregulation mean capillary pressure was kept essentially unchanged to judge from a maintained isovolumetric level indicating that the locally induced vasodilatation was within the precapillary section During these periods of lowered pressure and slightly lowered intestinal blood flow constrictor fibre stimulation produced almost the same vascular response as at normal arterial pressure so long as arterial pressure level was not below approximately 60 mm Hg (Fig 4) However at very low arterial pressures when resting blood flow was more distinctly reduced the neurogenic vasoconstrictions became gradually more depressed Here also it sometimes seemed as if the postcapillary resistance and capacitance response was somewhat better maintained than the precapillary resistance Whenever the isovolumetric level was not maintained during the stimulations tissue volume tended to increase

slowly, a situation similar to that seen in skeletal muscle (Lewis and Mellander 1962) When normal pressure and flow was re established the response to stimulation was usually rapidly restored to control

The extent of the capacitance vascular responses to constrictor fibre activation were deduced from the rapid phases of tissue volume change occurring simultaneously with the resistance changes at the onset and interruption of sympathetic stimulation (see Mellander 1960) If venous outflow pressure is kept very low a decrease in mean capillary pressure along with a neurogenic increase of the pre to postcapillary resistance ratio can so lower venous transmural pressure as to precipitate a collapse of the veins A profound expulsion of blood would then occur whether the venous smooth muscles were simultaneously activated or not In this situation passive elastic factors would overshadow to a considerable extent active venous contractions and in the venous collapse phase the active component would hardly reveal itself at all

On the other hand if the initial venous outflow pressure is raised so as to keep the entire venous section well distended an entirely different situation is present Even a drastic lowering of capillary pressure along with the resistance response would then not cause venous collapse even though a slight passive elastic recoil would be obtained In this situation most favourable circumstances for revealing an active venous contraction would prevail also because contractile elements are moderately stretched to start with

To test the validity of these theoretical considerations which will be more systematically discussed in another publication (Öberg 1964) the following simple test was performed in a few experiments The superior mesenteric artery was occluded first at a low venous outflow pressure and then when venous outflow pressure was moderately raised When the phase of passive venous emptying was completed the constrictor fibres were stimulated in both instances at 8 imp/sec and the additional amount of blood thus expelled from the intestinal vessels was measured As could be expected a low venous outflow pressure favoured passive elastic emptying and only little extra blood could be squeezed out from the collapsed vascular bed by the stimulation When venous outflow pressure was elevated the passive elastic emptying was small and nerve activation was able to expel nearly one third of the intestinal blood content It is therefore evident that when it is desired to analyse more exactly the extent to which a vasoconstrictor fibre discharge causes an active mobilization of blood from the thinwalled capacitance section this should be exposed to a moderately raised venous outflow pressure Since both active and passive effects on the venous side are highly important the capacitance responses to nerve stimulation were studied at both low (Fig 2) and slightly raised (Fig 3) levels of venous outflow pressure With a raised venous outflow pressure the capacitance response was a rapid decrease in volume at the onset of stimulation which was fairly well maintained for the duration of the stimulation At the cessation of stimulation the volume returned rapidly to or near the control level Increasing

the frequency of stimulation increased the magnitude of the respo With a low venous outflow pressure the effect of passive elastic recoil v a eadily demonstrable in addition to the active component by ■ greater exp sion of blood from the region With the appearance of autoregulatory escape there was a partial reversal of the volume change which was interpreted ι mainly a passive partial refilling of the venous compartment At the cessation of t mulation there was a transient increase in volume above the control level I u was interpreted as the effect of the release of constrictor fibre activity on the apaci tance vessels as well as a passive distention of these vessels due to an increased transmural pressure in association with the reactive hyperaemia

It was a general experience that the active contraction of the capacitance vessels expelled up to 10 to 15 per cent of the intestinal blood volume at a dis charge rate as low as 1 imp/sec The extent of the blood mobilization increased rapidly with increased stimulation frequency to reach practically maximal values with expulsion of 30 to 40 per cent of the blood content at 4 to 6 imp sec These figures agree closely with Mellander s findings (1960) on the veins of the cat s hindquarters and with Celander s findings (1954) on the extent of emptying of the splenic blood depots

With a slightly elevated venous outflow pressure (Fig 3) the rapid volume increase at the cessation of stimulation was approximately as large as the de crease at the onset of stimulation This indicates a well maintained capacitance response during stimulation in contra distinction to the resistance response

The general pattern of the responses of the various sections of the intestinal vascular bed was the same with all of the anesthetics used It was also in principle the same with or without the presence of adrenal medullary secretion in agree ment with observations in other vascular beds (Celander 1954) or whether atropine had been given or not

Discussion

As in all other autonomic neuro-effectors the pattern of response of the in testinal resistance and capacitance vessels to regional vasoconstrictor fibre ac tivation depends on the rate of fibre discharge and the number of fibres that are excited When all the vasoconstrictor fibres to the intestine are excited clearcut responses are obtained at frequencies as low as 1 imp/sec or even less Maximal effects in the steady state phase of constriction of the resistance and capacitance vessels are reached at impulse rates well within the physiological range i e up to ■ imp/sec (Folkow 1955)

The resistance response of the intestinal vessels to constrictor fibre stimula tion exhibits some very characteristic features For one thing it ■ very soon counteracted by a local vascular adjustment which has here been called an autoregulatory escape from the constrictor fibre influence Therefore an initial peak response occurs followed by an often far more moderate constrictor phase

which is then maintained at a fairly steady level At stimulation frequencies of 6 to 8 imp/sec and higher, the peak response may be great enough to suggest the occurrence of a transient widespread critical closure within the precapillary resistance section However the higher the discharge rate and the more pronounced the peak response the more powerful generally is the autoregulatory escape phenomenon This has the consequence that the flow resistance during the steady state phase of neurogenic vasoconstriction very seldom is more than about doubled and this maximum is reached at rates as low as 4 to 8 imp/sec In fact at still higher discharge rates this steady state phase of vasoconstriction tends to decline again The autoregulatory escape is a phenomenon characteristic of the intestine but not of resting skeletal muscle or skin in which the constrictor fibre effects are generally wellmaintained (see Folkow 1955) It is a truly physiological phenomenon not an experimental artifact (e g failure to maintain adequate excitation of the vasoconstrictor fibres) First even when there was a most pronounced autoregulatory escape with a minimally increased flow resistance in the steady state cessation of stimulation invariably produced a considerable increase in blood flow This reactive hyperemia was usually greater the more pronounced the autoregulatory escape This observation shows the presence of vasoconstrictor fibre influence on intestinal resistance vessels during the whole period of stimulation although evidently counteracted by some local dilator mechanism Second the constrictor fibre effects on the vessels and on the precapillary sphincters (see companion paper) were generally well maintained throughout the stimulation period Third it was noted in a few experiments in which basal vascular tone and normal flow autoregulation to pressure alterations gradually vanished that the autoregulatory escape to sympathetic stimulation gradually became less pronounced and ultimately virtually disappeared In such situations constrictor fibre stimulation was able to evoke wellmaintained constrictor responses of the resistance vessels and in addition to this a continuous volume decrease indicating an increased pre to postcapillary resistance ratio producing a net inward filtration of fluid from the extravascular space The pattern of response thus became similar to that seen in skeletal muscle and skin in these exceptional cases These latter findings also suggest that the autoregulatory escape is a phenomenon mainly related to changes in smooth muscle tone of the precapillary resistance vessels This interpretation is further supported by the fact that passive elastic collapse of the venous capacitance section occurring during the initial peak response of the resistance vessels during vasoconstrictor fibre stimulation was overcome in parallel with the appearance of the autoregulatory escape as judged by the partial recovery of the blood volume lost from the region Fig 2)

The experimental evidence therefore strongly suggests that the autoregulatory escape phenomenon takes place somewhere within the precapillary resistance section as does the autoregulatory adjustment to primary blood pres-

sure changes (Johnson and Hanson 1962) It seems probable tha se two types of locally induced vascular adjustments have at least to som tent a common denominator The exact mechanism is not known but it m e that specific shunt vessels are opened in connection with autoregulato cape Some type of blood flow redistribution seems to take place within the e time during these circumstances because with prolonged high frequency s ation such a powerful autoregulatory escape took place that the result was ghtly increased average blood flow during the stimulation period (e g Fig at 8 imp/sec) In spite of this a pronounced reactive hyperemia occurred on e sa tion of stimulation Had this slightly increased blood flow during the stimula tion period been evenly distributed to all the nutritional vessels of the inte tine no debt in blood supply would have been present and it would not then have been followed by a reactive hyperemia It might be that the splanchnic stimula tion increased the metabolism of the intestine However this is hardly likely since the results were the same when atropine was given which tends to depress both motility and secretion There was never any evidence of secretory or motility response to the sympathetic stimulation if anything motility tended to become supressed

Another interesting feature of the resistance response in the steady state phase is the fact that capillary pressure was kept surprisingly constant In a situation of unchanged arterial and venous pressures this implies a most exact mechanism for keeping the pre to postcapillary resistance ratio unchanged despite the fact that both resistances do increase The nervous influence *per se* on the vessels seems to increase this ratio during the peak phase of the constrictor response to judge from the often profound drop in pressure within the venous compartment at this time However the capillary pressure appears to return to the prestimulatory level by the time that the phase of autoregulatory escape is established It is therefore likely that it is this latter local mechanism rather than the primary constrictor fibre influence which is responsible for the exact maintenance of the capillary pressure level In fact the constrictor fibre in fluence when taken alone seems to be similar to that in the skeletal muscle that is it increases the pre to postcapillary resistance ratio and hence tends to lower mean capillary pressure (Mellander 1960)

It is surprising that when arterial pressure is allowed to increase during neurogenic vasoconstriction of the intestine with a constant venous outflow pres sure capillary pressure nevertheless tends to remain constant during the steady state phase of the constriction Here therefore the pre to postcapillary re sistance ratio must increase just precisely enough to keep the capillary pressure the same

Such a well controlled maintenance of intestinal capillary pressure despite wide variations in constrictor fibre activity seems to be a great advantage if not a necessity for the intestine considering its enormous capillary surface area (Folkow Lundgren and Wallentin 1963) This large surface area is of great

advantage for secretion and absorption However, were it unprotected against shifts in mean capillary pressure a massive edema of the mucosa could be formed rapidly with even a small increase in mean capillary pressure On the other hand in skeletal muscle it appears that neurogenic influences on the Starling equilibrium are utilized for fluid shifts between the intra and extravascular compartments (Mellander 1960 Öberg 1963)

Maximal steady state responses of the capacitance vessels were obtained also at discharge rates as low as 4 to 6 imp/sec at which frequencies 30 to 40 per cent of the intestinal blood content could be expelled by active contraction alone Since venous pressure is normally quite low and the veins are highly distensible any lowering of venous transmural pressure *e g* by an upstream resistance constriction will be expected to produce a pronounced passive emptying of the venous side which in most cases must then add to the active contraction As shown in the present study passive elastic recoil and venous collapse could be so pronounced during peak response of the resistance vessels, as to almost overshadow the direct constrictor fibre effect on the capacitance vessels a phenomenon that will be dealt with in more detail in another study (Öberg 1964) When however the experiment was so designed as to minimize the passive elastic element it could be shown that the capacitance response to constrictor fibre stimulation was in general more sustained and stable than the precapillary resistance response which was so markedly affected by the autoregular escape This difference in the maintenance of these two responses does not occur normally in resting skeletal muscles but can be seen for example during prolonged periods of reduced regional blood flow (Lewis and Mellander 1962) This phenomenon might explain why occasionally during intense sympathetic stimulation the capillary pressure level in the intestine tended to increase slightly with a resulting slow outward filtration The veins form not only the main part of the capacitance section they also have a small but important postcapillary resistance function In the intact organism there is an additional postcapillary resistance namely within the liver which appears to increase to some extent during sympathetic stimulation Any increase in this resistance would act in the direction of increasing mean capillary pressure in the intestine and lead potentially to edema formation

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The Effect of the Sympathetic Vasoconstrictor Fibres on the Distribution of Capillary Blood Flow in the Intestine

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Abstract

FOLKOW, B., D. H. LEWIS, O. LUNDGREN, S. MELLANDER and I. WALLENTIN. *The effect of the sympathetic vasoconstrictor fibres on the distribution of capillary blood flow in the intestine.* Acta physiol. scand. 1964. *61.* 458—466. — Experiments are described which show that a marked and well sustained reduction in intestinal capillary filtration coefficient (CFC) is induced by vasoconstrictor fibre stimulation. This decrease in CFC is considered to reflect a widespread closure of precapillary sphincters with an obstruction of the corresponding capillaries. India ink injections indicate that this neurogenic obstruction of capillary flow particularly affects the intestinal mucosa. The fact that this marked neurogenic reduction of capillary blood supply to the mucosa takes place concomitantly with an only moderate increase of intestinal blood flow resistance suggests that a considerable redistribution of blood flow takes place within the intestine. The hemodynamic changes produced by the vasoconstrictor fibres in the intestine are compared with the drastically different situation in the skeletal muscles. Some functional implications of these findings are briefly discussed, as well as the possibility that intestinal blood flow is diverted to submucosal shunt vessels.

In a previous paper (Folkow *et al.* 1964) the effect of graded vasoconstrictor fibre stimulation upon the intestinal resistance and capacitance vessels was described. As was obvious from this study the vasoconstrictor fibre effect on the intestinal precapillary resistance vessels is strongly counteracted by a local vasodilator mechanism. This causes an "autoregulatory escape" from the constrictor fibre influence, so balanced that in the steady state phase of constrictor fibre activity intestinal flow resistance is only moderately increased and the ratio between the pre- and postcapillary resistance is the same as before stimulation. The neurogenic influence on the capacitance vessels, on the other hand, appears to be fairly well maintained even during prolonged periods of constrictor fibre activity.

It was repeatedly observed especially during constrictor fibre stimulation at higher rates that the autoregulatory escape of the precapillary resistance vessels could be so pronounced that intestinal blood flow was hardly decreased at all in the steady state phase of constrictor fibre activity Sometimes it was even increased as compared with the prestimulatory level Nevertheless a profound reactive hyperemia usually followed the cessation of stimulation indicating that possibly a redistribution of intestinal blood flow had taken place during the stimulation so that some areas were actually in a state of relative ischaemia while others were overperfused

In the present paper results are reported which indicate that the vasoconstrictor fibres affect blood flow distribution within the intestine This was recorded by following the changes in blood flow resistance and in capillary filtration coefficient (CFC) as well as the distribution of intra arterially injected India ink before during and after constrictor fibre activation

Method

The experiments were performed on 25 cats anesthetized as described in the previous paper (Folkow *et al* 1964) which should also be consulted for the experimental technique used The method for estimation of CFC has previously been described by Folkow Lundgren and Wallentin (1963) In principle periods of graded increases of venous outflow pressure were induced intermittently so as to raise the mean capillary pressure a known extent Such a temporary rise in venous outflow pressure produces a biphasic increase in intestinal volume There is an initial rapid phase caused by venous distension which is followed by a slow but steady phase caused by an outward filtration in those capillaries which are open to blood flow Since there is good reason to assume that the constrictor fibres do not affect capillary permeability *per se* (*e g* Folkow 1955 Renkin and Rosell 1962) a decrease of CFC during constrictor fibre stimulation was considered to reflect a largely proportional reduction in the size of capillary surface area available for exchange or roughly in the number of capillaries perfused This would then imply that a constriction of at least some vascular sections had been induced which was strong enough to entirely interrupt blood flow in the corresponding series-coupled capillaries For convenience these vascular sections will be called precapillary sphincters but it should be stressed that this term is used only in a functional sense It does not mean that the sphincter function is necessarily located immediately proximal to the capillary entrance as a vascular closure in still more proximal sections of the arterial tree would have similar consequences for capillary perfusion and CFC

To visualize those parts of the intestine that might have become deprived of their blood supply by the action of the constrictor fibres India ink was injected i a in a number of the experiments in the control period during nerve stimulation both at the moment of peak resistance response and at the steady state response (see Folkow *et al* 1964) as well as after ended stimulation

For this purpose the intestinal preparation was divided into four or five segments and India ink was injected selectively into the desired segment via an i a cannula The circulation to the other segments was then obstructed temporarily by loose rubber shod clamps without damaging their vessels or vasomotor nerves It could be controlled that no damage was inflicted upon the vasoconstrictor fibres by this procedure since stimulation after this procedure was able to evoke the same extent of blood flow decrease as before obstruction of the vessels

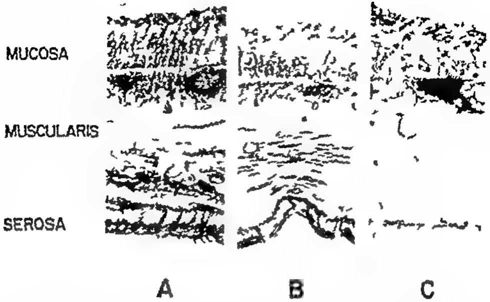

Fig. 2. Cat 3.0 kg. Chloralose. Microphotos of 100 μ frozen sections of intestinal wall extirpated immediately after in vivo close intraarterial infusions of India ink.
A. During resting conditions of the denervated gut. Note the extensive India ink filling of the mucosal vessels.
B. During peak response to unilateral vasoconstrictor fibre stimulation at 8 imp/sec. Note the blanching of the mucosa indicating a closure of its blood vessels presumably at the precapillary sphincter level.
C. During steady state response to unilateral at 8 imp/sec. Note that the India ink filling of the *mucosal vessels is more pronounced than in B but much less than in A.*

capacitance response on the other hand seems to be well maintained since the considerable reduction of intestinal blood content appears to be about the same during the entire stimulation period. Moreover, throughout this period of stimulation CFC was kept reduced to about half the prestimulatory level but as soon as the stimulation period was over, CFC increased to values higher than the prestimulatory level and was then well correlated with a fairly prolonged reactive hyperaemia. Therefore this in principle typical but *by no means especially drastic response to prolonged constrictor fibre* stimulation indicates that the nervous influence on precapillary sphincters like that on the capacitance vessels is well sustained even under circumstances where the neurogenic increase of *flow resistance is rapidly more or less abolished* because of the autoregulatory escape. The lower part of Fig. 1, taken from the paper of Cobbold *et al.* 1963, illustrates as a contrast the considerable difference in the relative effects of a constrictor fibre discharge on *flow resistance and* precapillary sphincters in the skeletal muscles. While the constrictor fibre influence on blood flow resistance is pronounced and well sustained in skeletal muscle, its precapillary sphincters exhibit a considerable escape. *In the intes-*

tine on the other hand it is the flow resistance that escapes from the influence of the constrictor fibres while their effect on CFC is relatively strong and well maintained It must be concluded that the competetive balance between the vasoconstrictor fibres and contrary directed local dilator mechanisms is markedly different in the two tissues with respect to the vascular sections that are mainly responsible of blood flow resistance and the number of perfused capillaries

Fig 2 shows the filling of the intestinal vessels with India ink during the resting state (A) during the peak (B) and steady state responses (C) to a unilateral splanchnic stimulation at 8 imp/sec This figure illustrates how a considerable number of small vessels, especially in the mucosa are well filled with India ink in the resting state while during the peak response to constrictor fibre stimulation there are only few of the smallest mucosal vessels visualized by an India ink injection In the steady state phase of the constrictor fibre influence the number of small mucosal vessels filled with India ink appears to be somewhat greater than during the peak response especially perhaps towards the luminal surface but definitely reduced as compared with the resting state In most cases the submucosal vessels too were well filled with India ink in this phase of constrictor fibre stimulation At the same time the intestinal flow resistance was hardly increased at all while measurements of CFC in other experiments indicate as mentioned that CFC could be expected to be much reduced at this stage

Admittedly India ink injections alone may give little information about the actual flow state but when the markedly reduced filling of mucosal vessels in the steady state phase of constriction runs parallel with a considerable reduction of CFC while almost the same blood flow passes the intestine it seems reasonable to suggest that a true blood flow redistribution has been induced by the constrictor fibres In the muscularis too flow seems to be reduced by the constrictor fibre stimulation because here also the India ink filling of the vessels was generally somewhat reduced though less dramatically so in most cases It thus appears that the flow is directed away especially from the mucosa which then means that some other intestinal section becomes overperfused either because of a widening of its vessels or because of an opening of new vessels It seems likely that this takes place mainly towards the submucosal section because often this intestinal part was well filled with India ink during stimulation when only little of the ink reached the mucosa proper These differences in India ink filling of the different intestinal tissue sections is often quite striking even on macroscopic inspection and provide additional information of interest In most cases the mucosa showed a patchy staining with India ink with sometimes completely blanched irregular but often wide areas in between especially when the injection had been performed during high frequency constrictor fibre stimulation The muscularis and the serosal surface on the other hand showed generally less striking differences from the prestimulatory state being usually somewhat paler without any distinct patchiness

Discussion

The present findings indicate that the vasoconstrictor fibres can produce a well sustained and in extent often profound diversion of blood flow from the intestinal mucosa to other parts of the intestine This diversion is characterized by a considerable reduction in the number of perfused capillaries while total blood flow through the intestine is relatively little decreased in the steady state phase of constrictor fibre influence These findings should be considered in relation to the specialized functions of the intestine with special regard to absorption and secretion events Both these functions call for a big blood flow and a big exchange surface between blood and the extravascular compartments within the mucosa This part of the intestine accordingly seems to be well safeguarded in this respect to judge from the big maximal blood flow capacity, the high CFC values of the intestine (Folkow Lundgren and Wallentin 1963) and the enormously dense network of mucosal vessels It is interesting to note that the vasoconstrictor fibres evidently exert a most powerful influence at some key point of the mucosal vascular supply so that profound and well sustained neurogenic obstructions of its capillary flow can be brought about It has been observed *e g* in the mucosa of the stomach (Arabehety *et al* 1959) that activation of vasoconstrictor fibres can produce similar most striking effects on the mucosal vessels in this part of the gastrointestinal tract though in this study no direct measurement of total blood flow through the stomach was performed *concomitantly*

The potentiality for such profound neurogenic reductions in capillary blood flow to the glandular sections of the gastrointestinal tract may be of great importance for gastrointestinal function as such Glands capable of profuse secretory responses are of course highly dependent upon a large blood supply especially then the plasma fraction since it delivers the raw material for the big secretion volumes Centrally induced vasoconstrictions may therefore in an indirect but highly efficient way suppress the activity of such glands simply by depriving them of the raw material necessary for their activity To what extent the vasoconstrictor fibres interfere with absorption seems to be less well known It would appear to depend to a great extent upon how much of the capillary network closest to the intestinal lumen becomes closed off from the circulation

The combination of profound neurogenic reductions of the number of perfused mucosal capillaries with an often surprisingly small reduction of total intestinal blood flow observed in the present experiments makes it likely that a true redistribution of flow takes place in such a way that the blood supply to some other intestinal layer may actually become enhanced during the activation of the constrictor fibres The question arises as to where such an increased blood flow could occur and whether it is then distributed over a capillary surface or at least partly diverted through shunt vessels which are opened

up or so affected as to exhibit a reduced flow resistance in connection with the constrictor fibre activation As mentioned earlier it appears that such an increase in flow may take place predominantly towards the submucosa though more precise information is not available in the present study In this connection it should however be mentioned that several morphological studies (e g Spanner 1932 Boyd 1952) have demonstrated the existence of fairly numerous huntlike vessels especially in the submucosal sections of the gastrointestinal tract, as well as in the salivary glands Moreover Walder (1952) found in a limited number of perfusion experiments on extirpated human stomachs that constrictor agents or sympathetic stimulation increased to an often considerable extent the number of intra arterially injected 40—200 micron glass spheres appearing in the venous outflow These most interesting findings seem to imply that some fairly wide bore vessels actually become opened up during a neurogenic vasocon trictor respon e It should then be recalled that according to Spanner (1932) the intestinal shunts have a peculiar arrangement with longitudinal smooth muscles an arrangement which appears to be fairly unique in smaller vessels Is it possibly so that these shunts become both shortened and widened during sympathetic activation concomitantly with the closure of mucosal sphincters so as to divert the blood flow from the mucosa to these shunts in the deeper structures? Further it may be questioned why particularly glandular structures characterized by an ability for voluminous secretions should be so well supplied with shunt vessels to judge from the mentioned morphological studies No doubt the shunts of these gastrointestinal glandular structures must be of functional significance in one way or another One theoretical possibility is that they erve as red cell channels which take the excess of red cell flow in a plasma skimming arrangement so designed that during secretion activity a preponderance of plasma flows in the mucosal vessels to serve as raw material for the secreting cells Upon constrictor fibre activation when the mucosal blood flow becomes largely obstructed hence depressing the secretion activity the shunts would take the main bulk of the flow Some possible consequences of such a hypothetical redistribution of intestinal blood flow in connection with the defence reaction are briefly discussed by Cobbold *et al* (1964) and these problems are currently under investigation in this laboratory Whichever the final solution of how the blood flow redistribution is arranged in detail it seems quite possible that the profound and wellsustained neurogenic obstructions of the mucosal vessels may have serious consequences in certain pathophysiological situations *c f* Texter 1963 The combination of an intense vasoconstrictor fibre activity a low perfusion pressure and changes in blood viscosity due to sludging etc might so severely interfere with the nutrition of the intestinal mucosa as to produce widespread necrotic lesions

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Blood Flow, Capillary Filtration Coefficients and Regional Blood Volume Responses in the Intestine of the Cat during Stimulation of the Hypothalamic 'Defence' Area

By

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Abstract

Cobbold, A., B. Folkow, O. Lundgren and I. Wallentin. *Blood flow, capillary filtration coefficients and regional blood volume responses in the intestine of the cat during stimulation of the hypothalamic 'defence' area.* Acta physiol. scand. 1964. 61. 467—475. — The effects of electrical stimulation of the hypothalamic 'defence' area on consecutive sections of the intestinal vascular bed has been studied in the cat. The response pattern observed was similar to that evoked by direct stimulation of the splanchnic nerves. Thus there was an initial pronounced vasoconstriction of the resistance vessels which, however, after 1—2 min declined and blood flow returned to near resting control values ("autoregulatory escape"). The capacitance vessels also constricted, expelling at most 30—40 per cent of the regional blood volume. In spite of the "autoregulatory escape" of the resistance vessels, capillary filtration coefficient (CFC) was markedly reduced throughout the whole period of stimulation, indicating a diminished capillary surface area available for fluid exchange. A nearly complete closure of the small intestinal mucosal vessels was illustrated also by India ink infusion performed before, during and after the hypothalamic stimulation. The functional significance of the response pattern with reference to the defence alarm reaction is briefly discussed.

The existence of an area within the hypothalamus of the cat from which the sympathetic vasodilator supply of muscle could be activated was shown by Eliasson *et al.* (1951) and this dilatation was associated with, among other things, a simultaneous vasoconstriction in the intestine. These findings, together with the suggestion that this hypothalamic area might act as a center responsible for reactions to states of emergency and alarm, were confirmed and extended by Abrahams, Hilton and Zbrozyna (1960) using both anaesthetized and conscious cats.

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The muscle dilator component of this response has been fairly fully investigated (Lindgren 1955 Abrahams *et al* 1960) but little attention has been directed towards the responses of the intestinal blood vessels It was considered that this might be of interest particularly as the vasomotor control of these vessels has been recently studied (Lewis Folkow and Mellander 1962 Folkow *et al* 1964 a b) and techniques have become available for assessing the activity of the capacitance vessels and the behaviour of capillaries through the reactions of the precapillary sphincters and determination of the capillary filtration rate The latter methods are based upon the work of Mellander (1960) in muscle and have been adapted for use with intestine by Folkow Lundgren and Wallentin (1963) Thus it has proved possible to study the behaviour of the intestinal blood vessels in more detail when activated from the defence area in the hypothalamus and to examine the concept that in such circumstances blood is diverted from the digestive tract into the vessels of skeletal muscle These results have recently been preliminarily reported (Cobbold *et al* 1963)

Method

Experiments were performed on 20 cats weighing 2·0—3·5 kg anesthetised i v with chloralose 40—50 mg/kg after preliminary induction with ether The methods used were broadly those described by Folkow *et al* (1963) Briefly after extirpation of the stomach omentum and spleen a section of jejunum usually weighing about 20—30 g was enclosed in a temperature controlled perspex plethysmograph The remainder of the small intestine and the large intestine was extirpated Great care was taken throughout to preserve intact the nervous connections of the jejunal segment To avoid any contribution to intestinal motility or to blood flow from the adrenal medullary secretion the right adrenal gland was generally removed or ligatured and the left adrenal was denervated In most experiments the vagal nerves were cut in the neck After heparinization the blood pressure was recorded from the right femoral artery using a mercury manometer Blood flow within the intestinal loop was recorded by diverting the blood flow from the superior mesenteric vein into an optical drop recorder the blood being then returned to the animal via a cannulated external jugular vein In some experiments the blood flow from skeletal muscle was simultaneously recorded by passing blood flowing from a cannulated femoral vein through a second drop recorder The occurrence of an atropine sensitive vasodilatation was then used to confirm that the correct hypothalamic area was in fact being stimulated

The volume of the jejunal segment within the plethysmograph was recorded continuously by a piston recorder and the venous outflow pressure could be adjusted to any desired level by means of simply raising the tube draining the outflow recorder the pressure increment so obtained could be measured on a water manometer coupled in the connection between the superior mesenteric vein and the drop recorder With the intestine in the plethysmograph a constant isovolumetric state implying an equality of outward and inward capillary filtration could easily be obtained by suitable adjustment of the venous outflow pressure The isovolumetric equilibrium was then disturbed applying a known increment of increased venous outflow pressure From the slope characteristics of the ensuing volume response the capillary filtration coefficient (CFC) could be calculated as ml fluid filtering mm Hg min 100 g tissue for full discussion of the method the paper of Folkow *et al* (1963) should be consulted

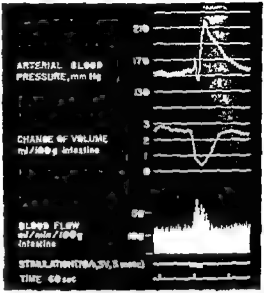

Fig 1 Cat 2 3 kg Chlo alose Effects of a brief stimulation of the defence area in the hypothalamus on blood pressure intestinal resistance and capacitance vessels The downward notch of the blood pressure recording reflects the activation of the sympathetic vasodilator fibres of skeletal muscle

For stimulation of the defence area the head of the animal was fixed in a Horsley Clarke apparatus and bilateral steel electrodes were inserted stereotaxically into the hypothalamic area known to give the typical emergency response in the circulation Square wave stimuli were delivered from a Grass stimulator Model S4C at 2—5 V pulse durations of 2—5 msec and at frequencies of 50—90/sec the parameters being selected to yield an optimal response At the conclusion of each set of experiments the position of the electrode was checked by dissection and staining

In some experiments the effect of hypothalamic stimulation on the small blood vessels within the intestinal wall layers was ascertained by carrying out such stimulations during local intra arterial infusion of India ink into only a segment of the jejunum The jejunal segment being so perfused was then immediately clamped and dissected free As controls other segments were then in a similar way injected with India ink while no hypothalamic stimulation was performed their vessels being exposed only to the resting sympathetic discharge Still other segments were injected with India ink when their vessels had been maximally dilated with isopropylnoradrenaline All these jejunal segments were frozen and cut using a freezing microtome and the extent of filling of the small vessels with India ink was then determined microscopically

Results

Fig 1 shows the effect of a brief (25 sec) stimulation of the defence area in the hypothalamus with a consequent pressor response the blood pressure rising by about 50 per cent and the flow resistance increasing to about 3 times that of the control The fact that the hypothalamically activated sympathetic vasoconstrictor discharge has a powerful effect on the capacitance vessels is shown by the immediate marked decrease in the volume of the intestinal loop That this initial decrease in volume is related to constriction of capacitance vessels

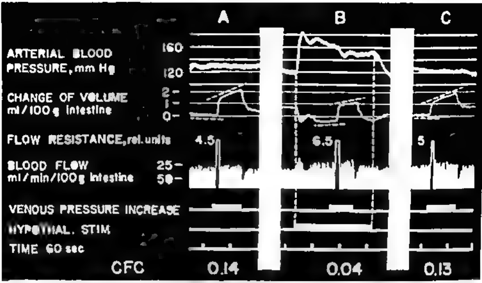

Fig 2 Cat 2 3 kg Chloralose Effects of stimulation of the defence area in the hypothalamus on blood pressure intestinal resistance and capacitance vessels and on capillary filtration coefficient (CFC) CFC estimated by increasing venous outflow pressure was determined before (A) during (B) and after (C) a hypothalamic stimulation Note the decrease in CFC during activation of the defence area

was shown by Mellander (1960) and in the present experiment the amount of blood expelled would correspond to roughly 25 per cent of the initial regional blood volume On cessation of stimulation the capacitance vessels immediately relaxed For details concerning the analysis of the capacitance response in the intestine see Folkow *et al* (1964 a)

Folkow et al (1963) found values for the resting blood flow of acutely denervated intestinal loops in cats of 40—60 ml/min/100 g at a pressure head of 100 mm Hg and these values or somewhat lower ones in general were approximated in most of the present experiments using innervated intestine This similarity in terms of flow resistance between innervated and denervated intestinal vessels is hardly surprising as low frequency sympathetic discharges seem to exert fairly weak effects on the intestinal resistance vessels in the steady state condition Folkow *et al* 1964 a) In the experiment illustrated in Fig 1 the resting flow was even somewhat higher around 85 ml/min/100 g and hypothalamic stimulation reduced this at peak constriction to some 35 ml min/100 g whilst simultaneously muscle blood flow increased and this increase could be abolished by atropine

The centrally induced decrease in blood flow in the intestine was however not persistent and tended during longer periods of stimulation to return to near the resting control value a phenomenon observed even when the constrictor fibres are stimulated directly at undiminished rate and intensity for a longer

Fig 3 Cat 3 kg Chloralo e Microphotographs of 100 μ frozen sections of the ntestinal all exturpated immediately after in vivo close intraarterial injection of Ind a ink
A. During resting condition with intact vasomotor fibre supply Note the moderate India nk fill ng of the mucosal vessels
B During stimulation of the hypothalamic defence ala m r action ar a. Note that hardly any India ink now reaches the mucosa indicating an almost complete closure of ts blood vessels, presumably at the precapillary sphinct r level
C. During intraarterial infusion of isopropyl oradrenaline Note the intense India ink stai ing of the mucosal vessels due to the vasodilator action of isopropylnoradrenaline

period (Folkow *et al* 1964 a) This phenomenon may be called an autoregulatory escape from vasomotor fibre influence The effect of sympathetic constrictor nerves on capacitance vessels on the other hand is in situations of relative ischemia generally better maintained throughout even prolonged activation periods in the vascular region of the skeletal muscles (Lewis and Mellander 1962) This seems to be true also for intestine when hypothalamic stimulation was continued for periods of 3 min and longer even if no procedures to cause relative ischemia were present

Hypothalamic stimulation with the parameters employed in these experiments can call forth a nearly maximal response from the vasoconstrictor fibres as judged from comparison with the effects seen during high frequency stimulation of splanchnic nerves (Folkow *et al* 1964 a) both in terms of the resistance and capacitance responses

Fig 2 illustrates the effects of a longer period of hypothalamic stimulation showing again the marked rise in blood pressure which occurs immediately With prolonged stimulation there was some tendency for the blood pressure to fall again after the initial increase of some 50 per cent above control values to a level of 25 per cent greater than control This effect might have been at least to a minor extent due to fatigue of or damage to the hypothalamic neurones from the stimulating current for following a period of rest whilst the response could be elicited again it was sometimes not quite as powerful as before it could however then be again fully obtained by shifting the position of the electrodes in the horizontal plane for a very short distance Fig 2 shows however that such a gradual failure of the hypothalamic stimulation could hardly alone be responsible for the pressure to fall and flow to be autoregulated in this instance because the constriction of the capacitance vessels persisted throughout the duration of stimulation

Capillary filtration coefficients were determined by increasing the venous outflow pressure and Fig 2 shows the typical response with this procedure This consists of an initial distension of capacitance vessels followed by a slower rising due to outward filtration of fluid Calculation from this slope gave values for the pre and poststimulatory CFC of 0 14 and 0 13 respectively whilst during stimulation the coefficient was drastically reduced then being only 0 01 The range of values for CFC obtained in the resting steady state in these experiments closely corresponded with that found by Folkow *et al* (1963) *i e* 0 1—0 15 ml/min/mm Hg/100 g tissue Upon stimulation of the 'defence area CFC always fell to very low values in some cases to less than 0 01 indicating a most extensive closure of the vascular sections that may be called precapillary sphincters *After the stimulation the precapillary sphincters were found to open almost* at once to give again normal or temporarily enhanced values for the intestinal CFC

The distinct and drastic closure of precapillary sphincters together with an identification of the actual capillary beds involved could be shown by dividing the jejunal loop into several segments with intact nerves and vessels after which infusion of a solution of India ink into the arterial supply of each segment could be performed One segment was perfused under resting conditions one during simultaneous infusion with isopropylnoradrenaline to produce maximal vasodilatation and the third during intense stimulation of the hypothalamic defence area Typical results are shown in Fig 3 The richly vascular mucosa can be seen in Section A showing resting intestine and even more clearly in C where with maximal vasodilatation the dense network of small vessels in the mucosal section is completely filled At B during the peak of hypothalamic stimulation the mucosal capillaries are seen to be almost completely shut off from the circulation and it was only with difficulty that India ink could be forced into the the intestinal capillaries On inspection of a bigger part of the intestine exposed to India ink injection during a hypothalamic stimulation the muscularis was generally fairly evenly stained while the mucosa showed wide areas that were quite blanched with small irregular patches of India ink filled vessels in between

Discussion

The results of the present studies entirely support the concept suggested by Eliasson *et al* (1951) and further by Abrahams *et al* (1960) that the hypothalamic area *from which sympathetic dilator fibres to* skeletal muscle blood vessles can be activated is the region responsible for an integrated defence reaction It would seem reasonable that an activation of muscle vasodilators should simultaneously involve an extensive activation of sympathetic vasoconstrictor fibres in regions such as the intestine whose function may be temporarily suspended during states of emergency and reactions of defence The immediate reactions of the intestinal vascular bed to hypothalamic stimulation demonstrate

that the most powerful and prolonged effect exerted in the defence reaction is a sustained constriction of capacitance vessels and well maintained closure of precapillary sphincters in the region of the intestinal mucosa The fact that around 30—40 per cent of the intestinal blood volume may be very rapidly expelled by prompt constriction of the capacitance vessels implies that at the beginning of an emergency state an auto transfusion of blood can be quickly delivered from the gastrointestinal tract to the heart to contribute to the augmentation of cardiac output to supply the dilated skeletal muscle vessel To gether with a constriction of the resistance vessels this accounts for the diminution of intestinal blood flow and blood volume which is observed The capacitance vessels however seem to maintain their constricted state better and this is in agreement with results obtained by direct stimulations of the sympathetic vasoconstrictor nerves to muscle and intestine (Lewis and Mellander 1962 Lewis Folkow and Mellander 1962 Folkow *et al* 1964 a) Even in skeletal muscle the precapillary vessels maintain their constriction less well than the postcapillary vessels and this seems to be even more apparent in the intestine This locally induced escape from the constrictor fibre influence in the pre capillary resistance vessels is very typical of the intestinal vascular bed and implies a sort of autoregulation Texter *et al* (1962) and Johnson and Hanson (1962) found that the autoregulation of intestinal blood flow in connection with pressure changes was localised in the arterial side of the vasculature and then to vessels less than 0 5 mm diameter i e to the precapillary vessels The same holds for the type of autoregulation that implies an escape of constrictor fibre influence as found by Folkow *et al* (1964 a) using direct stimulation of splanchnic nerves and as here reported, using stimulations within the hypothalamus

These similarities should however not be taken to imply that the autoregulatory precapillary dilatation following falls in blood pressure is exactly comparable to the autoregulatory escape following upon a constrictor fibre activation In fact it may be entirely different in nature depending upon which precapillary vascular sections are involved One obvious difference is that CFC is lowered during the autoregulatory escape from constrictor fibre stimulation but if anything increased in the precapillary autoregulation occurring as a response to a primary drop in arterial pressure (Folkow *et al* 1964 a)

It is further interesting that the sections of the mucosal precapillary vessels which determine the number of capillaries open to flow (precapillary sphincters) obviously react quite differently to constrictor fibre stimulation as compared with their functional analogues in skeletal muscle Stimulation of sympathetic vasoconstrictor fibres in the latter tissue causes no doubt initially a closure of many precapillary sphincters but this effect is only of a very temporary nature (Cobbold *et al* 1963) Within a minute or two CFC in skeletal muscles increases again and with maintained stimulation it generally exceeds its prestimulatory value This is attributed to the pronounced relaxing effect of metabolites accumulating in the muscle consequent upon the resulting flow

decrease and such a rapid increase in CFC is seen also when relative ischemia is produced by a partial arterial occlusion. CFC in the intestine, on the other hand, is kept reduced throughout the stimulation (Folkow *et al.* 1964 a, b).

Whichever the cause of the "autoregulatory escape" of the precapillary resistance vessels, which will relatively rapidly tend to curtail the initial blood flow decrease occurring during states of emergency, this flow supplies now only to a greatly decreased extent the intestinal mucosa. In other words, there seems in effect to be a sort of "shunting" of blood through other structures in the intestinal wall, a phenomenon which is at present being investigated in this laboratory.

The experiments with India ink infusion into the intestinal vessels indicate clearly that during the hypothalamic stimulation it is the mucosal precapillary sphincters which in wide areas must be quite closed, but India ink does to some extent penetrate the capillary bed of the submucosal structures. Quantitatively the influence of hypothalamically activated precapillary sphincters is considerable as seen by comparing the resting range of CFC at 0.1—0.15 to that during stimulation, being then reduced to 0.01—0.04. Thus there may be up to a tenfold decrease in capillary transfer during the time that the intestinal circulation is participating in a defence type reaction. Evidently the constrictor fibres may exert a most powerful and lasting effect on intestinal precapillary sphincters, mainly within the mucosa, while the intestinal resistance vessels rapidly escape from the constrictor fibre influence. Whatever the background of this peculiar difference between intestine and skeletal muscles in small vessel response to constrictor fibre activity, it may have some interesting consequences for gastrointestinal and liver function in a "defence" response. Such a considerable closure of mucosal capillaries in emergency states would tend to suddenly interrupt intestinal function when digestive activity is going on with secretion and absorption processes. The vascular response not only diverts blood volume and blood flow from the intestine but will indirectly suppress the metabolic requirements of these tissues by removing the blood supply necessary for secretion and absorption events. Furthermore, if it is so, as was discussed *e. g.* by Folkow *et al.* (1964 b), that a considerable fraction of the intestinal blood flow is diverted through opened shunt vessels during intense vasoconstrictor fibre activity, the oxygen delivery to the liver would still remain high in spite of an overall decrease of portal blood flow. This may prove to be of importance because in situations of violent exertions, as will usually ensue in the defence reaction, the liver becomes involved in an enhanced production of suitable fuel for the skeletal muscles and in the elimination of lactic acid.

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Reactions of the Different Series-Coupled Vascular Sections upon Stimulation of the Hypothalamic Sympatho-Inhibitory Area

By

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Abstract

FOLKOW, B., J. LANGSTON, B. ÖBERG and I. PREROVSKY. *Reactions of the different series-coupled vascular sections upon stimulation of the hypothalamic sympatho-inhibitory area.* Acta physiol. scand. 1964. *61.* 476—483. — A detailed study of the vascular responses induced from a previously described sympatho-inhibitory area in anterior parts of the cat's hypothalamus has been performed. The results indicate that topical activation of this area produces an inhibition of the tonic sympathetic activity not only to the heart and the resistance vessels but to the precapillary sphincters and the venous capacitance side as well. A truly generalized inhibition of sympathetic control of the entire cardiovascular system therefore seems to be produced, normally combined with an excitation of the vagal heart fibres.

The characteristic cardiovascular effects caused by this hypothalamic sympatho-inhibitory area are compared with the strikingly different response produced when the closely situated "defence reaction" area is activated, with a brief discussion of the functional significance of these two diencephalic integration centers.

In an earlier paper from this laboratory a hypothalamic structure with a marked inhibitory effect on tonic sympathetic activity was explored (Folkow, Johansson and Öberg 1959). It was then found that topical stimulation of this particular area with suitable stimulation characteristics evoked a profound blood pressure fall, due to a vasodilatation in all vascular circuits studied, and an inhibition of heart activity, revealed as an often considerable bradycardia. Since these events took place in vagotomized and atropinized animals also, the mentioned cardiovascular adjustments were interpreted as caused by an inhibition of the prevailing sympathetic vasoconstrictor accelerans fibre activity, though normally the vagal fibres to the heart seem to be activated too. A close

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ly similar response can be induced by stimulations in the cingulate gy r of the cerebral cortex the abovementioned hypothalamic structures probably being a relay station for such cortically induced circulatory adjustments (Löfving 1961) As the tonic constrictor fibre influence generally seems to be most marked on the vessels of the skeletal muscles the centrally induced overall inhibition of sympathetic activity often produces an increased muscle blood flow in spite of the drop in pressure

Such a centrally induced cardiovascular response pattern has a strong resemblance to that seen e g in emotional fainting in man As a working hypothesis it was therefore suggested that psychogenic reactions of this nature might normally be induced from the studied cortico-hypothalamic structure Since one of the more obvious components occurring in emotional fainting reaction in man seems to be a pooling of blood within the venous system in all probability due to a centrally evoked inhibition of the sympathetic discharge to the capacitance side of the circulation it was thought to be of interest to study the responses to topical stimulation of the hypothalamic sympatho-inhibitory area not only with respect to the resistance vessels but also to the capacitance vessels then utilizing a technique which permits a simultaneous study of the reactions of these two functionally different series coupled sections of the vascular bed

Methods

The experiments were performed on 9 cats superficially anesthetized with chloralose 30 to 40 mg/kg b w After exposure of the calvarium and drilling of a hole through the bone concentric bipolar electrodes insulated except at their tips were bilaterally orientated in the abovementioned hypothalamic area by means of a Horsley Clarke instrument Topical stimulation could be made by a Grass stimulator the intensity usually being 1—4 V with a pulse frequency of 30 to 80 per sec and a pulse duration of 1 msec

Vascular reactions were studied either in a skeletal muscle skin preparation usually the hindquarters of the animal or in the small intestine using techniques earlier described (Mellander 1960 Folkow Lundgren and Wallentin 1963) These papers should be consulted for full details concerning the preparation Briefly the tissue region to be studied is isolated from the animal except for its cognate artery and vein and its vasomotor fibre supply The organ is then enclosed in a temperature-controlled water tight plethysmograph by means of which a continuous volume recording of the organ could be accomplished When the hindquarter preparation was used the plethysmograph was filled with water while the plethysmograph used for the intestinal preparation was filled with Tyrode solution

Blood flow was continuously recorded by diverting the venous outflow from the tissue either through a drop recorder device operating an ordinate writer or through a modified Gaddum recorder The venous blood was automatically restored by means of a funnel connected to the jugular or inferior caval vein to the animal and so designed that the outflow from the recorder always matched the return of blood to the heart The height at which the venous outflow tubing was mounted above the tissue region determined the venous outflow pressure level which thus could either be kept constant or altered to any desired level when so needed by adjustments of the level of the outflow tubing The arterial inflow pressure was measured in the inferior mesenteric

artery when the hindquarter preparation was used and in one of the femoral arteries when the intestinal preparation was utilized The arterial inflow pressure could when so wanted be kept constant by means of adjustments of a screw clamp around the aorta, applied just proximally to the respective cognate arteries and proximal to the site of the arterial pressure recording Thus arterial inflow pressure venous outflow pressure total blood flow and changes in tissue volume of the studied organs were continuously recorded Changes in tissue volume reflect variations both in regional blood volume and in net transcapillary fluid transfer but these two separate parameters can in most circumstances easily be distinguished because of their different time courses Under these circumstances it is possible to follow quantitatively and continuously the reactions of the resistance vessels and the capacitance vessels in the vascular region studied as well as changes in the net transcapillary filtration considered to reflect changes in the pre m postcapillary resistance ratio and in the number of capillaries open to flow

In order to eliminate centrally induced vagal effects on the heart as well as accidental activations of the cholinergic vasodilator fibres to the vessels of the skeletal muscles both vagal nerves were cut in the neck and atropine 0 3—0 5 mg/kg was given intravenously In order to create a high initial sympathetic tone level and to avoid to some extent compensatory adjustments elicited from the carotid sinus baroreceptors when the sympatho-inhibitory area in the hypothalamus was stimulated both carotid arteries were as a rule clamped during the period of hypothalamic stimulation The animals were as a rule curarized with Flaxedil 2—3 mg/kg and artificial respiration was then administered in such a way that tidal volume and frequency was closely similar to the spontaneous breathing

At the end of each experiment the weight of the tissues was determined from which the approximate regional blood volume could be deduced on the basis of earlier estimations of the steady state blood content in the tissues studied (Mellander 1960 Folkow *et al* 1963)

Results

Fig 1 shows a recording from a representative experiment where the effect of hypothalamic stimulation upon vascular reactions in the small intestine was studied To start with the common carotid arteries were clamped At A a topical excitation of the hypothalamic sympatho-inhibitory area was performed with a stimulation frequency of 60 a voltage of 4 and a pulse duration of 1 msec The stimulation causes a blood pressure drop of about 60 mm Hg and in spite of the drop in perfusion pressure some increase of intestinal blood flow The flow resistance can thus be calculated to decrease to less than half the resting value indicating a marked dilatation of the resistance vessels It can also be seen from the volume recording that there is a rapid gain of tissue volume amounting to about 0 4 ml This rapid increase in tissue volume can be considered to reflect a dilation of the capacitance vessels (mainly the veins) leading to an increase in regional blood content corresponding to roughly 10 per cent of the calculated resting regional blood volume in the intestine Upon interruption of the stimulation there is a gradual restoration of blood pressure and tone of the resistance and the capacitance vessels In B the arterial inflow pressure to the region is mechanically reduced for a brief period to about the same level as that produced by the hypothalamic stimulation by adjustment of the

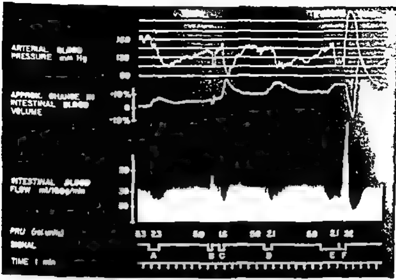

Fig 1 Cat 3 8 kg Chloralose 50 mg/kg The effects of stimulation (4 V 60 /sec) in the hypothalamic sympatho-inhibitory area upon the resistance and capacitance section of an intestinal vascular bed (A D and E) At F the stimulation intensity is suddenly changed from 4 to 7 V the electrodes still located in the sympatho-inhibitory structure At B the right vagus nerve is stimulated in afferent direction (8 V 20/sec) Both vagus nerves cut in the neck carotid arteries clamped bilaterally The animal is atropinized (0 3 mg/kg) curarized (Flaxedil 3 mg/kg) and artificial respiration administered Intestinal weight 54 g

screw clamp placed around the aorta As could be expected this procedure which lowers the pressure head and the transmural pressure within the intestinal vascular bed causes a decrease not only in blood flow but in regional blood content as well to judge from the small volume decrease This finding indicates that the increase of tissue volume obtained at A should have been even more pronounced in case the arterial inflow pressure had been kept constant

In C an afferent vagal stimulation (8 V 20 per sec 1 msec) was performed As can be seen from the recordings this procedure induced reflex vascular reactions almost identical to those obtained by hypothalamic stimulation though being established more promptly that is a blood pressure fall is obtained and simultaneously a considerable reduction of the flow resistance is induced combined with a rapid gain in tissue volume indicating a dilatation of the capacitance vessels

In D and E hypothalamic stimulations are again performed with virtually the same results as in A However in E the stimulation strength was suddenly increased from 4 volts to 8 volts while the electrodes were still placed within the hypothalamic sympatho-inhibitory area (F) It has repeatedly been observed that such an increased stimulation intensity which causes a more widespread hypothalamic activation tends to produce an excitation of the defence

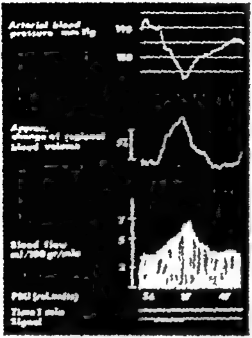

Fig 2 Cat 3 9 kg Chloralose 40 mg/kg The effect of stimulation in the hypothalamic sympatho-inhibitory area (4 V 60 sec) upon the resistance and capacitance vessels in a skin muscle preparation (hindquarter of the cat) Both carotid arteries clamped the vagus nerves cut bilaterally in the neck Atropine (0.2 mg/kg) Flaxedil (3 mg/kg) and artificial respiration administered. Weight of the hindquarter preparation 1 700 g

eaction area also as this integration centre is situated just ventrally to the sympatho-inhibitory area in the hypothalamus at a distance of only 1—1 5 mm. It can be seen that this more extensive hypothalamic stimulation where the defence pattern dominates the cardiovascular events produces the reverse effects compared with that obtained when the sympatho-inhibitory area was more selectively activated. Thus an abrupt increase in blood pressure up to 230 mm Hg occurs accompanied by a marked reduction in blood flow and in tissue volume. At the peak response the intestinal blood flow resistance increased in fact some 10 times while about 1 2 ml blood corresponding to some 30—40 per cent of the regional blood volume was expelled from the intestine findings which are in good agreement with recent observations by Cobbold et al 1963 and 1964. This last stimulation of the hypothalamus clearly demonstrates the range of the vasomotor adjustments that can be centrally induced from specific diencephalic areas morphologically closely situated but functionally quite differently organized. Note the marked reactive hyperemia ensuing upon cessation of the stimulation.

In several of the experiments the hypothalamic stimulation was continued for a longer period to make possible an evaluation of the changes in the filtration events over the capillary membrane. To perform such a study it is necessary to reach a steady state in the vascular adjustments where during a period of hypothalamic stimulation the new level of tone of the resistance and capacitance vessels can be maintained fairly stable. Shifts in tissue volume occurring

under such conditions must then be ascribed to changes in net transcapillary filtration. It is then also possible to determine the capillary filtration coefficient (CFC), a parameter which reflects the size of the capillary surface area available for blood flow and hence filtration. Therefore CFC can be considered to provide information concerning the tone of those parts of the small vessels which are generally called the "precapillary sphincters" (see Folkow and Mellander 1960; Cobbold *et al.* 1963 a). Studies of this type, which were only performed on the intestine vessels as the precapillary sphincters of the muscle vessels appear to be only little influenced by the constrictor fibres (Cobbold *et al.* 1963 a), indicated that CFC in the intestine often increased by about 20 to 30 per cent upon excitation of the hypothalamic sympatho-inhibitory area. In other words, beside the centrally induced dilation of the resistance and capacitance vessels of the intestine, more capillaries seemed to be opened for blood flow, presumably because of an inhibition of the sympathetic discharge to those vascular smooth muscles also, which control blood flow through individual capillaries in the intestine.

The results described here were essentially similar in direction and magnitude in all experiments, whether the intestine or the muscle-skin region was studied, as long as effective stimulation of the hypothalamic sympatho-inhibitory area could be achieved. Fig. 2 shows recordings from one experiment, where the vascular reactions in a skeletal muscle-skin preparation were followed during hypothalamic stimulation. It can be seen that excitation of the hypothalamic sympatho-inhibitory area causes a reduction of the flow resistance of about 80 per cent and an increase of tissue volume corresponding to about 10 per cent of the calculated regional blood volume.

It therefore seems justified to conclude that this diencephalic structure causes an inhibition of the tonic sympathetic vasoconstrictor fibre activity in a generalized fashion [illegible] not only affect the resistance vessels [illegible] different parallel coupled circuits, as shown earlier (Folkow *et al.* 19[illegible]), but [illegible] the whole all the different series coupled sections in a given vascular bed which are normally controlled by the vasoconstrictor fibres.

Discussion

Investigations concerning the influence of cortical and hypothalamic [illegible] tures on the [illegible] side of the vascular bed are so far very [illegible] majority of earlier studies dealing with cardiovascular adjustments [illegible] from [illegible] mesencephalic autonomic centers have been [illegible] with [illegible] blood pressure and heart rate, and only in a [illegible] parameters like regional blood flow changes have [illegible] The technique used in the present [illegible] the possibilities for [illegible] detailed study of the peripheral [illegible] that can be [illegible] morphologically and functionally [illegible]

hypothalamic area It was thus possible to confirm the findings of earlier studies (see Folkow *et al* 1959 Lofving 1961) which showed the existence of a fairly restricted area in the hypothalamus from which often marked inhibitions of the tonic sympathetic vasoconstrictor accelerans fibre discharge could be induced The present study further shows that this centrally induced inhibition of the sympathetic vasoconstrictor activity in all probability is a generalized one affecting all sympathetically innervated series coupled sections in a vascular bed Thus beside the *reduction in heart rate and in the flow resistance* of all parallel coupled circuits normally controlled by a tonic vasoconstrictor fibre discharge the activation of this hypothalamic structure also produces a relaxation of the capacitance vessels with a consequent tendency of peripheral pooling of blood as well as a relaxation of the precapillary sphincters in such tissues where these vascular sections are influenced by the constrictor fibres This overall inhibitory cardiovascular pattern should be contrasted to such a highly differentiated excitatory response as that which can be induced *e g* from the defence reaction area This latter response pattern is comprised by an activation of sympathetic vasodilator fibres to the resistance vessels in the skeletal muscles with no significant engagement of their constrictor fibres while the corresponding capacitance vessels and virtually all vascular sections of other circuits like those of the intestine the kidney and the skin appear to be markedly affected by a concomitant activation of their constrictor fibres (see *e g* Jonas 1960 Folkow Mellander and Öberg 1961 Cobbold *et al* 1963 b 1964 Engl Johansson and Lofving 1962)

Recent studies (Lofving 1961) have made it probable that the hypothalamic sympatho-inhibitory area is a relay station for cortically induced inhibitory effects on the medullary vasomotor center It was also suggested by Lofving that these cortico hypothalamic structures might be responsible for such centrally induced cardiovascular reactions that in man can provoke emotional fainting partly because the observed changes in heart rate in blood pressure and in flow resistance in the various vascular circuits appeared to be closely similar The present finding that an inhibition of the sympathetic tonic control of the venous side forms part of this centrally evoked response is in agreement with such a hypothesis as venous pooling appears to be a dominant feature in the abrupt cardiovascular change taking place in emotional syncope

The present experiments also show that the reactions of the various consecutive vascular sections induced from the hypothalamic sympatho inhibitory area closely resembled those evoked reflexly by *e g* afferent vagal stimulation Evidence has earlier been presented by Lofving (1961) to indicate that the cortico-hypothalamic sympatho-inhibitory effects and the depressor reflexes evoked from cardiovascular baroreceptors seem to be relayed within the same part of the medullary depressor area The close similarity even with respect to details in the vascular responses produced by hypothalamic and by vagal depressor stimulation favours this view

On the whole the range of effects on the venous side that can be induced from higher autonomic structures seems to be considerable The often marked inhibition of the sympathetic influence on venous tone accomplished by stimulation of the hypothalamic sympatho inhibitory area should here be contrasted to the intense venoconstrictor effects that could be induced from the closely situated defence centre as was recently shown also by Cobbold *et al* (1963 b 1964) The importance of a wide range of nervous control of venous tone as reflexly governed by cardiovascular receptors via the medullary vasomotor centre is well recognized and makes possible adequate adjustments of venous return to the heart in the maintenance of circulatory homeostasis The present study indicates that still higher cardiovascular autonomic centres in the intact organism presumably engaged in more complex efferent patterns expressing *e g* changes in alertness in emotional equilibrium etc can considerably affect the hemodynamically important venous side with all the consequences such an influence may have on venous return to the heart and hence on cardiac output

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Nature of Shunt Path and Active Sodium Transport Path through Frog Skin Epithelium[1]

By

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Abstract

USSING H H and E E WINDHAGER *Nature of shunt path and active transport path through frog skin epithelium* Acta physiol scand 1964 *61* 484—504 — Sodium movement across frog skin epithelium follows two pathways a transport path and a shunt path for passive movement of Na as well as other ions Results of microelectrode experiments indicate the existence of a discontinuous electrical potential gradient within the epithelium Sodium apparently diffuses across a sodium selective barrier located just underneath the cornified layer into cells of the stratum spinosum and across desmosomes through stratum germinativum cells towards pump sites in the inner cell membranes of stratum basale cells However stratum spinosum cells also may pump sodium into epithelial interspaces which might communicate rather freely with the inside medium Doubling of osmolarity of the outside solution by addition of urea to Na_2SO_4-Ringer's leads to marked increase in the magnitude of the shunt conductance of sodium and sulphate ions Potential differences measured by means of microelectrodes indicate that the increment in passive ion movement under such circumstances involves intercellular channels

It has been demonstrated by Koefoed Johnsen and Ussing (1956 1958) that the response of the frog skin to variations in the ionic composition of the bathing solutions could be largely explained if its is assumed that 1) the outward facing boundary of the epithelium is permeable to sodium lithium and chloride but virtually impermeable to potassium and sulphate 2) that the inward facing boundary is permeable to potassium as well as to small anions like chloride whereas it shows practically no passive permeability towards sodium and 3) that the inward facing boundary possesses an active transport mechanism which pumps sodium ions towards the inside bathing solution

Experiments by Hoshiko and Engbæk (1956) and Engbæk and Hoshiko (1957) in which the intraepithelial potential was measured by means of Ling

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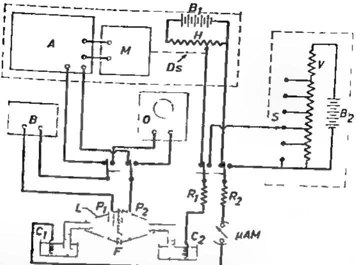

Fig 1 Diagram showing frog skin chamber and electrical circuits
A Amplifier B Variable bias voltage (± 200 mV) B_1 and B_2 Batteries C_1 and C_2 Current electrodes D Driveshaft F Frog skin H Helipot L Lucite chamber M Servomotor O Oscilloscope P_1 and P_2 Potential electrode (Calomel half cells with saturated KCl bridges) R_1 and R_2 10 Ω resistances S Step resistance consisting of calibrated resistances for increasing or decreasing the current through the skin in 5 equally large steps. V variable resistance

Gerard type microelectrodes were in good agreement with the above mentioned hypothesis since two abrupt potential steps were observed as the microelectrode was advanced through the skin Recently Whittembury and Herrera (1963) showed by iontophoretic deposition of dye spots at the points where the microelectrode potentials were measured that the potential steps were indeed located within the epithelium of skins of *Bufo marina*

It is clear that if the anions present could not penetrate one or both boundaries of the epithelium and if the sodium and potassium selectivity of outer and inner boundary were absolute the skin potential would be given by

$$E = RT/F\,(\ln\,(Na_o/Na_i) + \ln\,(K_i/K_s))$$

where R is the gas constant T the absolute temperature and F the Faraday Subscripts o c and s refer to outside solution cellular compartment and inside bathing solution respectively

Skins treated on the outside with 10^{-4} M $CuSO_4$ and skins in contact with solutions containing only the slowly penetrating sulphate ion usually give high potentials (sometimes as high as 160 mV) and such skins behave towards changes in the Na/K ratio of the bathing solutions in good agreement with the above equation Not infrequently however skins even in sulphate show much lower potentials especially during late winter and early spring Such skins usually exhibit less than the expected changes in potential when the sodium concen

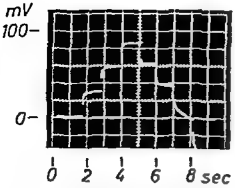

Fig 2 Potential responses to stepwise changes in current through the skin Na_2SO_4 Ringer's on both sides Abscissa Each division 2 sec Ordinate 5 divisions 100 mV At zero time the skin is short-circuited. The current is then reduced to zero in three steps of 7 9 μA cm each, after which the current is again increased in four steps — 100 mV means inside positive relative to outside

tion on the outside or the potassium concentration on the inside is varied These observations would be understandable if the skins in question were leaky to one or more ion species in other words if the skin battery were more or less shunted The shunt could be due to leakage of anions (chloride or sulphate) but leakage of sodium through the K selective inner membrane or potassium leakage through the Na selective outer membrane would also contribute to the shunt All ions present might produce a further effect in case the interspaces between the epithelial cells present a leakage path across the epithelium If the magnitude of the shunt is determined it should be possible to estimate the true value of the electromotive force of the skin battery Combined with microelectrode studies this approach should make it possible to locate and characterize the individual potential steps contributing to the total electromotive force

The experiments of Engbæk and Hoshiko (1957) as well as those of Whittembury and Herrera (1963) seemed to indicate that the potential steps were located at the inner and outer boundary of the basal cell layer of the epithelium Recent observations of Koefoed Johnsen (in preparation) indicate however that the sodium permeable potassium impermeable boundary must be located closer to the outside of the skin This was indicated by the observations that 1) all potassium of the epithelium exchanges readily with K^{42} added to the inside bathing solution and 2 the specific activity of K in slices from the outer half of the epithelium increased just as fast as that of the inner half under such conditions Thus a reinvestigation of the magnitude as well as the localization of the potential steps seemed indicated

Methods

Estimation of the shunt

First method The method depends on the assumption that the conductance of the sodium transport path is negligible compared to the conductance of the shunt path if the outside bathing solution is made sodium free Thus we shall assume that the skin d c resistance becomes equal to the shunt resistance when all sodium of the outside sol

Table I Composition of the Ringer's solutions used (m M/l)

	Na_2SO_4-R	K_2SO_4-R	$MgSO_4$-R	Urea Na_2SO_4 R
Na_2SO_4	55.5	—	—	55.5
K_2SO_4	0.95	56.5	0.95	0.95
$NaHCO_3$	2.38	—	—	2.38
$CaSO_4$ ($2H_2O$)	0.89	0.89	0.89	0.89
$KHCO_3$	—	2.38	2.38	—
$MgSO_4$	—	—	55.5	—
Urea	—	—	—	100.0

tion is replaced by potassium The choice of potassium as the "non penetrating" cation is of course rather arbitrary The cation in question should however fulfil the following requirements

1) It should have the same valency as sodium so that the ionic strength and the osmotic pressure of the outside solution is not changed by the procedure 2) It should be unable to penetrate into the epithelial cells from the outside 3) It should be physiologically inert under the conditions of the experiment Potassium seems to fulfil these requirements reasonably well The D C resistance was estimated from the change in potential resulting from a sudden change in the D C current running through the skin The current was applied through the usual current bridges of the frog skin chamber (Ussing and Zerahn 1951) as modified by Koefoed Johnsen Ussing and Zerahn (1952 By aid of a special potential divider circuit shown in Fig 1 it was possible to change the current in five equally large steps chosen to cover the region between zero potential and the open circuit skin potential Two large resistances (0 1 megohm each in series with the skin made the current through the skin independent of the skin resistance and the polarization resistance of the electrodes During the resistance measurements the potential bridges of the frog skin chamber were connected to the input of a double beam oscilloscope (Tektronix) through 10 megohm attenuation probes giving 10 times attenuation of the signal The sweep was set at 0 5 cm/sec and the potential scale corrected for attenuation) was usually 20 mV per cm Fig 2 gives the typical picture of the potential steps resulting from changing the current from 23 7 μA/cm² to 0 μA cm² and back in steps of 7 9 μA/cm² The outside medium is sulphate Ringer's solution (see Table I for composition) Fig 3 was obtained on the same skin after replacement of all Na of the outside medium by K In this case each step was 3 3 μA/cm² As far as Fig 3 is concerned the interpretation presents no difficulties The steps are about equally large with sharp shoulders and the size of the steps does not depend on whether the current is being increased or decreased Thus for practical purposes the resistance is ohmic With sodium present in the outside medium (Fig 2) the situation is not quite as simple As long as the current is decreased the steps are always well defined and reasonably similar at different current strengths The D C resistance can be estimated by extrapolating the horizontal (or nearly horizontal parts of the trace back to the time of current change When the current is increased from the situation of open circuit towards the state of [illegible] tial ("short-circuit [illegible] the first steps usually are also well defined and similar [illegible] obtained with decreasing current But after two or three steps for most skins [illegible] of instability is reached which makes a measurement of the D C resistance impossible If the current is maintained constant the potential runs through a sequence of changes

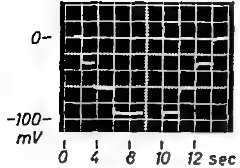

Fig 3 Potential responses to stepwise changes in current through the skin K_2SO_4 Ringer s outside Na_2SO_4 Ringer s inside
Abscissa Each division [illegible] sec Ordinate 5 divisions 100 mV At zero time the spontaneous potential is close to zero An ingoing current is now applied in 4 steps of 3 9 μA/cm each and again reduced stepwise to zero − 100 mV means inside negative relative to outside

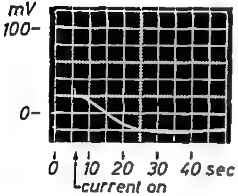

Fig 4 Potential response to sudden application of the current previously found to Short circuit the skin Na_2SO_4 Ringer s on both sides
Abscissa Each division = 5 sec Ordinate Each division 20 mV At zero time the potential is 102 mV At arrow application of short-circuit current 30 μA/cm

looking rather like damped oscillations (see Fig 4) This phenomenon may be related to the so called action potential of the frog skin (see Finkelstein 1961) In order to escape the complications resulting from this transient all resistances were calculated from potential steps following a decrease in current If this is done most skins can be considered as nearly ohmic resistances even with sodium ions present in the outside medium

From the data given in Fig 2 and 3 it can be seen that the resistance of the skin is much higher (7 950 ohms cm²) with potassium outside than with sodium (3 030 ohms cm²) in agreement with the theoretical expectation A similar finding has been reported by Ito (1962) As soon as potassium replaces sodium in the outside medium the resistance starts increasing reaching a constant high level after 15 to 30 min When sodium sulphate Ringer s is re introduced the lower level of the resistance usually comes back faster generally within 5 min Why the establishment of the high resistance with potassium outside takes longer time is uncertain Possibly the sodium of the epithelial cells has to be leached out or transported inward before the conductance of the sodium transport path approaches zero

In all experiments of the above type sulphate was used as the anion because this makes it possible to keep the volume of the epithelium constant throughout the experiment (compare MacRobbie and Ussing 1961 This point is of major importance because swelling always decreases the skin resistance whereas shrinkage increases it (Ussing in preparation)

Second method The method depends on the assumption that 1) the sodium pump itself has no conductance (in other words it is impossible to force sodium ions through

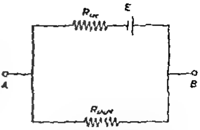

Fig 5 Equivalent circuit representing electrical properties of the frog skin A and B points of contact between epithelium and outside and inside bathing solution
E_0 Electromotive force of the active transport system, R_{act} D C resistance of active transport path R_{shunt} Resistance of shunt path through the skin

the pump in a reversible way by means of an electrical field) 2) That the passive flow of sodium and the major anions of the Ringer s solution (here sulphate) gives the major contributions to the shunt The partial shunt conductances of sodium and sulphate can then be obtained from the isotopically measured passive fluxes determined under conditions where the back diffusion is negligible Routinely this was done by clamping the skin potential at 100 mV (inside positive) and measuring sodium efflux and sulphate influx, respectively with sulphate Ringer s on both sides Except for a possibly small contribution of exchange diffusion of sulphate this method must measure the sulphate shunt The shunting effect of sodium may be larger than the value obtained from the sodium efflux because some sodium ions which may have passed the inward facing boundary could have been pumped back into the inside medium These would contribute to the shunt without contributing to the measured efflux However since we have reasons to believe that the inward facing boundary is rather tight to sodium the source of error due to recirculation of this ion may not be too serious Ordinary back diffusion is not likely to play any role From the flux equation (Ussing 1949 Teorell 1949) it can be seen that with identical Na concentrations on both sides and a potential difference of 100 mV sodium back diffusion is about two per cent and the back diffusion of a divalent ion like sulphate 0 04

The arrangement used for clamping of the skin potential is shown in Fig 1 It is a modification of the automatic short-circuitter designed by Mullins (1958) Flux measurements were carried out using the methods previously described (Ussing and Zerahn 1951)

Theory for estimation of shunt The relationships permitting the calculation of the true electromotive force of the skin battery can be visualized by aid of the equivalent circuit shown in Fig 5 E_0 represents the electromotive force of the system and can be tentatively assumed equal to the sum of the sodium diffusion potential at the outer boundary and the potassium diffusion potential at the inner boundary Even if the sodium pump were electrogenic the expressions derived would, however still be valid R_{act} is the D C *resistance in series with the electromotive force and can be assumed to consist of the* diffusion resistance for sodium at the outer and for potassium at the inner boundary of the active transport path Finally R_{shunt} is defined as $1/k_{shunt}$ where k_{shunt} is the sum of the D C conductances of all ion flows tending to short-circuit the skin battery" According to Ohm s law the D C resistance of the skin R would then be determined by the equation

1) $1/R = 1/R_{act} + 1/R_{sh}$

When the skin is in the open circuit condition that is if no current is running through any outer connection between inside and outside bathing solution the skin battery is only shorted through R_{shunt} and this inner current is given by

2) $I = E/R_{shunt}$

where E is the skin potential as measured between terminals A and B (inside and out

side bathing solutions) This current must be equal to the current running through the transport path For the closed circuit consisting of the transport plus the shunt path we have

3) $I = E_0 / (R_{shunt} + R_{ser})$

Combining 2) and 3) we obtain

4) $E/R_{shunt} = E_0 / (R_{ser} + R_{shunt})$

Both R and E are readily measurable Thus since it is now also possible to estimate R_{shunt} equations 1) plus 4) enable us to estimate the true electromotive force of the skin E_0) as well as the series resistance of the active sodium transport path (R_{ser})

Methods used in microelectrode studies on frog skin

Experiments were performed on the abdominal skin of the brown frog Rana temporaria Frogs were pithed and the skin then removed and washed in sodium sulphate Ringer's solution

The skin was mounted in the apparatus for measuring changes in the thickness of the frog skin epithelium as described by MacRobbie and Ussing (1961) In this apparatus the skin is tied to a plastic ring in such a way that a cup is formed (surface area of exposed skin 7 1 cm²) with the outside of the skin facing upwards The inside of the skin rests on a thin glass wool pad in a shallow lucite dish Adequate oxygenation is assured by a regular flow of solution through the glass wool pad facing the inside of the skin The experiments were performed at room temperature (about 20 C)

Ling Gerard type microelectrodes (Ling and Gerard 1949) were filled with 3 M KCl by boiling under reduced pressure when used in experiments which did not involve iontophoretic dye injection In those experiments in which electrode sites were marked with lithium carmine was deposited iontophoretically (Mitarai 1960 and Whittembury and Herrera 1963) For this purpose 1 2 g lithium carmine were dissolved in 100 ml 3 M KCl (made alkaline by addition of KOH to pH 9) centrifuged and filtered Since dye aggregates form rapidly in this solution it is necessary to fill each single microelectrode immediately before use In this procedure the microelectrode is filled though a very thin long hand-drawn micropipette inserted through the large opening of the microelectrode Insertion of the filling capillaries was usually possible to within a few microns distance from the tip opening of the electrode Because of the high pressure necessary to inject through the filling pipettes a motor driven pump had to be used Microelectrodes were selected on the basis of electrical resistance (2—20 megohms) and tip potential (less than 10 mV) Tip potential and tip resistance were checked in the respective bathing solution immediately before and after each impalement by the method of Adrian (1956) The microelectrodes were inserted in a lucite electrode holder containing a calomel half cell as previously described (Whittembury and Windhager 1961) The indifferent electrodes consisted of polyethylene tubes filled with Ringer agar in contact with another calomel half-cell and were placed either in outer or inner bathing solution The recording instrument was a model B D C vacuum tube voltmeter (Keithley Instruments Cleveland Ohio When continuous graphs of the potential profile were desired the voltmeter was connected to a Tektronix double beam oscilloscope Graphs were obtained either by drawing on transparent paper or by the use of a polaroid camera (Tektronix type C-12 camera

The microelectrode holder was mounted in a micromanipulator and the impalement of the skin done under 100 × magnification using a Leitz dissecting microscope The skin was penetrated in oblique fashion by advancing the microelectrode in steps of about 1 to 5 μ Measurements of potential differences were only accepted when on advance of the electrode the potential change occurred abruptly and when the new difference remained stable within 3 mV for at least 20 sec On numerous occasions poten

Table II

	Total shunt conductance (measured with K_2SO_4 Ringer's outside) $ohm^{-1}cm^{-2}10^{-5}$	Partial shunt conductance of Na and SO_4 from ionic flux $ohm^{-1}cm^{-2}10^{-5}$	Partial conductance in per cent of total shunt conductance
Na-efflux[1]	10.6	6.[illegible]	58.5
	5.8	2.0	34.6
	11.1	4.8	43.3
	19.6	7.0	3[illegible].7
	11.5	6.[illegible]	54.0
	17.3	8.[illegible]	47.4
	20.0	9.[illegible]	48.0
Mean			46.0 ± 3.6
SO_4 influx	18.5	[illegible]	30.3
	12.8	3.5	[illegible]7.4
	1[illegible].[illegible]	4.9	39.2
	11.[illegible]	4.9	43.8
	14.3	4.2	29.4
Mean			34.[illegible] ± 3.5

Skin potent al clamped at 100 mV ins d pos tive during flux measurements.

tial measurements were continued for several minutes Only when photographic recording was required for illustration purposes was the potential measurement sometimes terminated after shorter periods by advancing the microelectrode through the skin Upon impalement of the skin from th out de unstable potential differences negative to the outside and inside solution ere observed These corresponded to imilar observations by Engbæk and Hosh ko 1 [illegible]7 Hoshiko 1961) and Whittembury and Herrera (1963) The electrode site gi ng these negative potential differences were found by the latter authors to be localized in th corn fi d layer of the skin In the present study no systematic invest gation of this phenom non has been attempted but in agreement with Whittembury and Herrera 1961 it was noted that essentially no electrical resistance could be measured bet er the t of thes unstable electrical potentials and the outside solution In all nstan es r wh h th microelectrode potential was measured with respect to both ins d a d o t d solution t as found that the sum of the two readings approximated th total skin pot ntial than ± 5 mV

Dye labelling of mi roel trode r cording site was done iontophoretically Th electrical ci cu t used for th purpose was essentially the same as that described by Wh tt m bury and Herrera 1963 To obta n informat on as to the site of the dye deposit w th th kin the t hn que f Ma Rob e and Ussing (1961) was applied The skin assembl was transferred from the tag of th dissecting microscope onto the stag of a b nocul microscope a d observed th ough a Leitz water immersion lens in the out de bath solution bject mag if ation 50 eyepiece magnification [illegible] The [illegible] listance from p gm nt granules of xanthophores just underneath the basal [illegible] to th dye d pos t to 10 μ thickness) and to the outside surface of the kin [illegible]

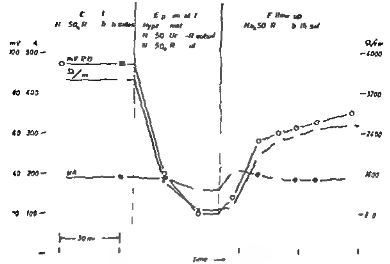

Fig 6 Effect of urea added to outside solution on potential short circuit current and resistance of frog skin bathed with Na_2SO_4 Ringer s on both sides

fence) was measured using the calibrated finefocusing control of the microscope The distances of the dye deposit from the xanthophore layer are given as per cent of total epithelial thickness as measured along the vertical axis through the dye spot Optical readings were reproducible to $\pm$ 1 μ

The total skin potential was measured with a high impedance null reading millivolt meter (Radiometer Copenhagen) For this purpose polyethylene tubes filled with Ringer agar of the appropriate composition served as potential electrodes connecting the respective bathing solutions and calomel half cells In experiments in which skins were short-circuited a similar arrangement was used as that described by MacRobbie and Ussing (1961)

The composition of the various Ringer solutions used in the present study is given in Table I

Results

Estimation of the shunt

Table II shows comparative data on total shunt conductance as measured by the first method in which all sodium in the outside medium had been replaced by potassium and on partial conductances of sodium and sulphate obtained by the second method from measurements of ionic fluxes The total shunt conductance shown in column 2 was estimated in 12 frog skins (K_2SO_4 Ringer s outside Na_2SO_4 Ringer s inside) and amounted to a mean value of 13 8 ohm^{-1} cm^{-2} 10^{-}

The third column gives the partial shunt conductances obtained from ionic flux measurements on the same skins In each experiment the isotope flux periods bracketed the period with K_2SO_4 Ringer s on the outside The isotope flux used for calculation was obtained as the mean of the values before and after

Table III Effect of addition of urea to outside

	Partial conductance of Na and SO from ionic flux $\Omega^{-1}cm^{-2}10^{-5}$		Total conductance from $\Delta I/\Delta E$ $\Omega^{-1}cm^{-2}10^{-5}$	
	Before urea	30 after urea	Before urea	30 after urea
Na-efflux	2 4	3?2	17 1	10?
	4 2	37.5	22.2	115
	5 1	51 0	54 7	150
	5 4	10 8	21 4	39 4
SO influx	5 0	26.8	16 7	83 4
	5 4	24 1	16 7	1?5
	5 4	>28	?3.3	111

the sodium free period The mean partial shunt conductance of sodium was 6 3 ohm^{-1} cm^{-2} 10^{-5} and that of sulphate 4 6 ohm^{-1} cm^{-2} 10^{-5} Comparison of the mean fractional partial conductances of the two ion species (see column 4) indicates that sodium contributes about 46 per cent and sulphate 34 per cent of the total shunt conductance

In order to assess whether the shunt is mainly intercellular or intracellular certain observations on skins treated on the outside with hypertonic solutions appear to be relevant It has been demonstrated by Ussing and Andersen (1955) that addition of glucose urea and certain other substances to the Ringer s solution bathing the outside of an isolated toad skin reduced its electrical resistance dramatically Frog skins exhibited similar response and urea was especially effective (Ussing 1960) Results of a representative experiment are shown in Fig 6 After control measurements with sodium Ringer s solution bathing both surfaces of the skin the osmolarity of the outside solution was approximately doubled by the addition of urea Immediately after urea had been added both the potential difference across the skin and the skin resistance fell precipitously whereas the short circuit current — which is a measure of the rate of active sodium transport — remained virtually constant A gradual return of potential and resistance towards the starting values took place during the follow up period with Na_2SO_4 R on outside

That the dramatic increase in conductance produced by urea resulted from an increase in both sodium shunt conductance and sulphate conductance is shown in table III Measurements of partial conductance of sodium and sulphate before and after addition of urea to the outside medium indicated that the partial conductance of both ion species increased up to about 10 times the control values Comparison of the increments in partial conductances of sodium and sulphate with the increments in total conductance after addition of urea makes it apparent that the increase in total conductance was due to increased leakiness of the skins to both ion species

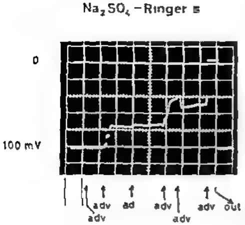

Fig. 7. Potential profile. Record goes from left to right. Indifferent electrode on inside. Microelectrode first records total skin potential 102 mV. On impalement (adv.) a short lasting break in the record results corresponding to unstable negative potentials observed in most skins during penetration of the stratum corneum. Upon further advance a stable potential plateau (– 78 mV) is seen which is not altered significantly (– 76 mV) by next advancement. Deeper penetration — after some potential fluctuations — results in plateau of – 50 mV. The microelectrode is advanced still further and the potential drops to + 2 mV (not significantly different from zero). Upon removal of the tip the full skin potential reappears.

Microelectrode studies

Potential profile experiments in skins bathed in Na_2SO_4 Ringer's

Seven skins bathed in sulphate Ringer's solution had total skin potentials of 80—130 mV (outside negative). Twenty seven microelectrode impalements were done. In sixteen of these profile pattern with only one potential plateau were encountered. The mean potential difference with respect to the inside bath amounted to — 64.3 mV ± 2.8 (SEM). Eleven impalements showed more than one potential plateau: two plateaus were found in seven punctures, three in three, and four different potential plateaus were observed only once. In every instance in which more than one level was found, each successive plateau was less negative than the preceding levels (see Fig. 7). In these experiments (Fig. 8) two groups of potential plateaus could be discerned: a first group with a mean potential difference of — 87.4 mV ± 3.1 (SEM) and the other with an average value of — 54.7 mV ± 1.9 (SEM). Infrequently (3 observations) a third group was found with a mean plateau of — 29.3 mV. Mean potential differences in the first and second group were statistically different (p less than 0.001).

Dye labelling experiments in skin bathed in Na_2SO_4 Ringer's

The sites used in twenty three stable potential measurements (9 frog skins) were marked by means of lithium carmine spots. The total skin potential averaged 129 mV, the thickness of the epithelium had a mean of 70.2 μ. All potential measurements were done with the indifferent electrode placed in the inside bathing solution. As shown in Fig. 9 the most negative intraepithelial potential plateaus were encountered in the stratum spinosum which corresponds to about 50 to 90 per cent of the total thickness of the epithelium. In this part of the skin there is a decrease in potential negativity with decreasing distances of the labelled sites from the pigment layer. This trend is absent in the inner

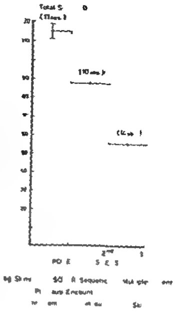

Fig 8 Grouped potential plateaus observed in all microelectrode p ofile impalements (total skin potential — 116 mV) First group (mean — 67 4 mV) includes 2 plateaus found in one single impalement second group (mean 54 7 mV) 5 plateaus observed during 2 penetrations A thi d group of 3 potential plateaus (25—30 and 33 mV) is not included in the d agram Dif ference between averages of first and second group (— 3? 7 mV) is highly significant (p < 0 001)

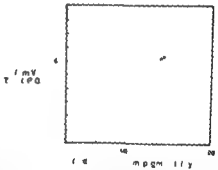

Fig 9 Site of microelectrode pot ntials in f og sk n ep thelium Microelect od potential in p cent of total skin potential plotted on the or dinate site of potential measu ments as relati e distance from p gment lay n per cent total th ckness of epith l um on the abscissa

half of the epithelium Apparently potential plateaus in the stratum germina tivum show no further decline but cluster around a mean value of 46 per cent of the total skin potential (corresponding to — 59 mV in this series of experi ments) Much less negative plateaus comparable to the three observations in profile penetrations in sulphate Ringer skins were localized close to the base ment membrane The significance of these potential differences remain un known however since they might be due to electrical artifacts during penetra tion of the layer of reticular fibers situated underneath the basement membrane One potential measurement of — ▮ mV was localized within the corium in dicating that the corium is practically equipotential with the inside bathing solution

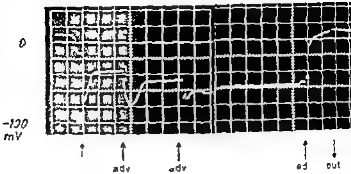

Fig 10 Tote tia pr fil Record tarts on the left side interruption in record after 50 sec indicates charge ... Total skin potential + 10 mV During impalement 3 stable potential plateaus were ... d — 37 — 47 and — 58 mV) Notice unstable negativ potentials between plateaus

Potential ... experiments in skins bathed in Na_2SO_4 Ringer s on the inside and $MgSO_4$ Ringer s on the outside

Six frog skins were used in experiments in which the inside bathing solution was of Na_2SO_4 Ringer s and the outside solution $MgSO_4$ Ringer s The mean total skin potential after equilibration amounted to + 5 mV with reference to the inside bath indicating reversal of the skin potential (indifferent electrode in inside bathing solution) Out of 40 potential profiles 13 showed single plateaus whereas 2 to 4 plateau values were observed in 27 penetrations (see Fig 10 for a representative experiment) Potential differences between multiple plateau values in single profile were less pronounced and almost always reversed in sign when compared with skins bathed in Na_2SO_4 Ringer s on both sides the average maximal difference (outer plateau inner plateau in any single profile) in $MgSO_4$ skins amounted to + 9 8 mV ± 3 9 (SEM), a value statistically different from zero ($p < 0.001$) This increase in negativity may be compared with the average maximal drop in potential in Na_2SO_4 skins of — 30 3 mV ± 3 2 SEM) $p < 0.001$) Fifty five measurements of potential difference in which the indifferent electrode was kept in the outside bath gave an average plateau potential of — 60 3 mV ± 1 6 (SEM) indicating that with reference to the outside the microelectrode potentials were reversed in sign when compared with sodium-sulphate skins Referred to the inside bath the mean potential plateau amounted to — 55 4 mV ± 1 9 (SEM) 26 observations In the majority of observations advance of the *microelectrode* from the outside resulted in the measurement of increasingly more negative potential differences in contrast to Na_2SO_4 skins where the highest negativities were observed just underneath the cornified layer

Almost invariably negative transients (20 to 40 mV) were observed during advancement of the microelectrode see Fig 10) Whereas the negative ...

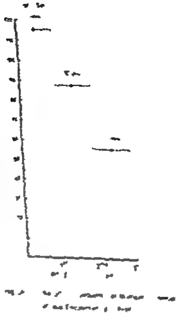

Fig. 8 Grouped potential plateaus observed in an microelectrode profile impalements (total skin potential − 116 mV). First group (mean − 7 4 mV) includes 7 plateaus found in one single impalement, second group (mean 34 mV) 5 plateaus observed during 2 penetrations. A third group of 3 potential plateaus (? − ?) and ? m) is not included in the diagram. Difference between averages of first and second group (− 37 7 mV) is highly significant (p < 0.001)

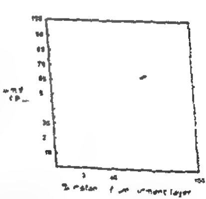

Fig 9 S e of microelectrode potentials in frog skin epithelium. Microelectrode potential in per cent of total skin potential plotted on the ordinate of potential measurement as relative distance from pigment layer in per cent total thickness of epithelium on the abscissa.

half of the epithelium. Apparently, potential plateaus in the stratum germinativum show no further decline but cluster around a mean value of 46 per cent of the total skin potential (corresponding to — 59 mV in this series of experiments). Much less negative plateaus comparable to the three observations in profile penetrations in sulphate Ringer skins were localized close to the basement membrane. The significance of these potential differences remain unknown however since they might be due to electrical artifacts during penetration of the layer of reticular fibers situated underneath the basement membrane. One potential measurement of — 2 mV was localized within the corium indicating that the corium is practically equipotential with the inside bathing solution.

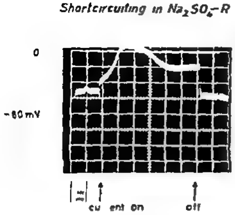

Fig 12 Microelectrode potential during short circuiting Potential difference of — 56 mV with respect to inside solution recorded initially on the left Frog skin short-circuited by sudden application of 25 V/cm Total skin potential not recorded on oscilloscope Current on and "off refer to short-circuit current

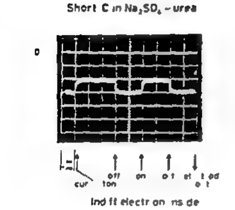

Fig 13 Microelectrode potential during short circuiting of frog skin bathed in hyperosmotic urea Na_2SO_4 Ringer s on outside Na_2SO Ringer s on inside Potential difference of — 60 mV with respect to inside solution recorded initially on the left of the oscillogram Frog skin short-circuited by sudden application of 35 μ V/cm Current on and off refer to short circuit current Notch in record of microelectrode potential on far right indicates (open) total skin potential of — 30 mV obtained on retraction of microelectrode into outside solution

urea experiments the average maximal difference between multiple plateaus (8 profile impalements) amounted to 34 6 mV ± 4 1 SEM a value significantly different from zero p < 0 001)

Short-circuiting experiments in Na_2SO_4 Ringer s

Three frog skins were used in which the mean total potential difference was 133 mV short-circuit current 32 μA cm² A total of 20 successful impalements was obtained The average non-shorted plateau potential was — 68 9 mV with the indifferent electrode in the inside bathing solution Sudden application of the previously measured short-circuit current resulted in a decrease of the negative potential plateau to an average stable potential of — 19 8 mV range + 4 to — 42 mV A representative record is shown in Fig 12 application of the short-circuit current results in a rapid fall in microelectrode potential which is followed by a slow decline and overshoot until a steady plateau is reached after about 20 sec In contrast to this is the very rapid return of the potential to its original value on breaking the short-circuit current

Short-circuiting experiments in skins bathed in urea Na_2SO_4 Ringer's outside and Na_2SO_4 Ringer's on the inside

Two skins when first mounted in sulphate Ringer's on both side had a short circuit current of 35 $\mu A/cm^2$ and a total skin potential of 140 mV After equilibration with Na_2SO_4 urea Ringer's on the outside the short circuit current remained essentially unchanged whereas the skin potential declined to 90 mV Ten successful penetrations were obtained in which the average intraepithelial potential plateau was — 57 0 mV before short circuiting On short circuiting the plateau values declined in negativity The mean potential plateau was then — 38 4 mV significantly more negative than the short circuited plateaus in the absence of urea ($p < 0\ 001$) Fig 13 shows that in urea skins the observed potential drop (transient) was very rapid both on "make and break of current This is in striking contrast to isotonic Na_2SO_4-experiments in which slow depolarization and overshoot were always observed after the initial rapid drop in potential

Discussion

Estimates of the magnitude of the shunt conductance indicate that in Na SO skins sodium seems to contribute about 46 per cent and sulphate 34 per cent to the total shunt conductance The fact that even in sulphate Ringer's this shunt can be appreciable at certain times of the year especially late in winter and spring goes a long way to explain why low potentials and less than theoretically expected responses to ionic substitutions are sometimes seen When corrected for the effect of the shunt even such poor skins usually have an electromotive force of more than 140 mV

A marked increase in conductance is brought about by the presence of hyperosmotic urea Na_2SO_4 Ringer's on the outside of the frog skin This drop in skin resistance results from an increased leakiness of the frog skin for sodium and sulphate ions To investigate whether the increase in conductance takes place in the shunt or in the active transport path hypertonic urea experiments were performed using the "first method in which all sodium in the outside medium was replaced by potassium In one experiment on addition of urea to the outside the shunt resistance dropped from 24 000 ohms per cm^2 to 3 000 ohms per cm whereas the total skin resistance dropped only from 3 300 ohms per cm^2 to 2 080 ohms per cm^2 Apparently urea treatment of the skin leads to decreased resistance in the shunt path rather than in the active transport path Further support for this view is provided by the fact that active transport of sodium remains virtually unaffected a condition which would be highly unlikely if the transporting cells were made thoroughly leaky by urea If on the other hand the leakage were due to an increased shunt between cells the situation would be understable This possibility was investigated by means of the microelectrode approach and the results are evaluated later in this discussion

The results of micropuncture experiments on skins in sodium sulphate Ringer's confirm the experimental findings of Engbæk and Hoshiko (1957) and of Whittembury and Herrera (1963) in those instances in which only two potential steps were encountered during a single impalement of the skin In about one third of the microelectrode penetrations performed in the present study multiple potential plateaus were found In these the first potential differences were nearly always more negative with respect to the corium than those found later during the impalement In attempting to understand the significance of this observation it became necessary to obtain information as to the histological site of the more negative (mean — 87 mV) potential plateaus Dye labelling and subsequent observation of the living isolated skin by water immersion microscopy showed that the inner half of the frog skin epithelium is approximately equipotential at an average potential of — 60 mV with respect to the inside medium Since the average thickness of the epithelia used in the present study amounted to 72 μ one might conclude that this potential difference prevails over a distance of about one to two cell layers from the basement membrane *i e*, the stratum germinativum Potential plateaus more negative with respect to the inside solution were located within the stratum spinosum This finding combined with the observed pattern of potential distribution indicates the existence of a discontinuous potential gradient between the outermost cells of the stratum spinosum and basal cells of the stratum germinativum Since cells in the outer layers of the stratum spinosum are flattened to a considerable degree the chance of obtaining stable intracellular microelectrode potentials is greatly diminished It is therefore not surprising that high and low potential plateaus are not encountered with about the same frequency and that multiple plateaus are a relatively infrequent occurrence in any given profile impalement The view that cells within the stratum spinosum maintain intracellular potentials of the magnitude and sign usually observed in living cells is supported by Wingstrand's finding (unpublished observations) of an abundance of mitochondria in stratum spinosum as well as in stratum germinativum of frog skin

One possible explanation of the discontinuous electrical gradient in frog skin epithelium would be a series arrangement of the various cell layers where movement of sodium ions from outside to inside might be linked with the observed potential jumps To test this hypothesis measurements were performed on $MgSO_4$ skins (Na_2SO_4 Ringer's inside) in which the direction of the intraepithelial movement of sodium is reversed Such skins are characterized by the absence of active transport of sodium ions The total skin potential is diminished or even reversed in sign and there is an increase in the electrical resistance of the skin Microelectrode impalements of $MgSO_4$-skins invariably showed a reversal of sign of the outer potential step With respect to the outer medium the intraepithelial potential differences were negative (— 60 mV) in contrast to skins bathed in sodium sulphate on both sides This finding is in accord with the frog skin model of Koefoed Johnsen and Ussing (1958) which assumes the

outward facing cell membranes to be selectively permeable to sodium ions However, it should be pointed out that otherwise negligible degrees of permeability of the outer membranes to other ions may be unmasked by the absence of sodium ions (vide equation of Goldman Hodgkin Katz Goldman 1943 Hodgkin and Katz 1949)

In line with the model of a series arrangement of cell layers the direction of the electrical gradient was reversed in $MgSO_4$ skins as compared to Na_2SO_4 skins With no sodium in the outside medium first voltage readings encountered on impalement from the outside to be less negative than subsequent potential plateaus In $MgSO_4$ skins sodium is not actively transported but leaks towards the outside solution Thus a reversal in the direction of transepithelial sodium movement goes parallel with a reversal in the direction of the intraepithelial electrical gradient It seems therefore logical to link the movement of sodium ions from the outside solution through the epithelial layers toward the pumping sites in the inner membrane of basal cells with the discontinuous potential gradient found in sodium sulphate skins According to this view sodium ions would encounter more than one barrier on their way through the skin The first barrier might be the outer cell membrane of the outermost cells within the stratum spinosum This could account for the first potential step with respect to the outside solution of about + 30 mV Further boundaries must be situated between this site and the basal cells As is well known neighboring epithelial cells are connected by numerous desmosomes Recently Fawcett (1961) has reinvestigated the desmosomes which had originally been described by Schaffer (1927) Fawcett s studies have shown that desmosomes are not true cytoplasmatic cell bridges but points of cell contact in which membranes can be demonstrated in electromicrographs On the basis of these morphological findings it is natural to assume that the desmosome membranes may act as resistive but possibly selective path to the movement of sodium ions

Evidence has been presented earlier in this study that an increase in tonicity in the outside solution caused by the addition of urea results in a considerable increase in the efflux of Na and in the influx of SO_4 Simultaneously, a marked drop in skin potential occurs with little or no change in short circuit current The main path for ion leakage under these circumstances could be transcellular or intercellular Results obtained in microelectrode experiments allow to discriminate between these possibilities

If the high efflux of Na and influx of SO observed under conditions of urea treatment were transcellular pronounced depolarization of the inner membrane potential should result However cells do retain their negative potential with respect to the inside solution under these circumstances In such experiment the epithelial volume as estimated by the MacRobbie Ussing technique (1961) is not different from that under control conditions except for [illegible] shrinkage immediately after addition of urea (Using unpublished [illegible]) that no net accumulation of SO takes place Thus it may be concluded that

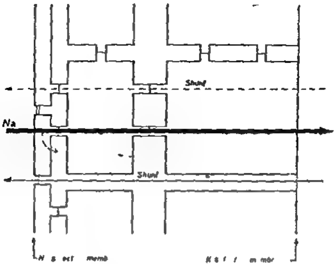

Fig. 14. Schematic diagram of frog skin epithelium.

the increased efflux of Na and influx of SO_4 pass through the epithelium via intercellular channels.

Short circuiting of frog skins bathed on the outside with sodium sulphate urea Ringer's (Fig. 13) does not produce the same pattern of intraepithelial potential transients as that seen in the absence of urea (Fig. 12). Since partial short circuiting conditions already prevail in urea skins before application of current, the abrupt depolarization without slow component or overshoot (indicating the absence of major change in ionic conductivity) is in agreement with the view outlined in the previous paragraph. The fact that intraepithelial potentials during short circuiting were more negative (by about 20 mV) than those observed in isotonic sodium sulphate skins might be due to a relative increase in sulphate conductance of the outer barrier induced by urea. Urea apparently increases the permeability of some boundary located at the outer face of the skin, governing the access to the "cellular path" as well as to the "shunt path", although the shunt path is much more affected.

Some of the features of sodium movement through the skin may be visualized in a schematic diagram shown in Fig. 14. Adjacent cells are always connected by "false cell bridges" or desmosomes and although the bridges are intersected by special membranes, which can be seen in electromicrographs, these membranes might conceivably have a much higher permeability to sodium than cell membranes proper. The sodium selective barrier is thought to be situated just underneath the skin cornified layer, the potassium selective boundary just outside the basement membrane. A discontinuous electrical potential gradient appears to be present within the epithelium, with abrupt drops in electrical negativity from outside to inside. If so, then extensive interchange of sodium be

tween cells and interspaces becomes highly unlikely It is probable that the intra epithelial potential jumps are due to the passage of sodium ions from outer cells via desmosome membranes to pumping sites in the innermost cell membranes. On the other hand the return path of sodium, and in general the shunt path for all passive ions should be thought to go partly between cells This would be particularly true when hypertonic urea solution is present on the outside of the skin In regard to the pumping sites which previously were thought to be confined exclusively to the inner membrane of the basal cell layer it is now conceivable that cells in the stratum spinosum also participate directly in the active transport of sodium by pumping the ions out into interspaces which may communicate rather freely with the inside bathing solution Towards the outside the interspaces are probably more or less closed by terminal bars (compare Farquhar and Palade 1963)

One of us (E. E. W.) is grateful for the hospitality shown by the members of the Institute of Biological Chemistry during his stay in Copenhagen The authors are indebted to Drs. [illegible] and Giebisch for the loan of some equipment used in this study and to Mr Poul Hansen, Mrs Ida Henningsen and Mrs Le la Tolsoe for valuable technical assistance

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Adrenergic Nerve Terminals in the Human Fallopian Tube Examined by Fluorescence Microscopy

By

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Noradrenaline (Brundin 1964 c) and adrenergic nerve terminals in the rabbit oviduct (*Brundin and Wirsén 1964*) are mainly confined to the circular musculature of the isthmus which suggests autonomic influence upon a sphincteric function of the rabbit isthmus recently described (Brundin 1964 a).

In this preliminary report the morphological basis for a similar function of the human isthmus *is demonstrated*.

A highly specific fluorescence method for histochemical demonstration of monoamines including noradrenaline in adrenergic nerve terminals (Falck 1962, Falck *et al.* 1962) was applied to Fallopian tubes obtained by hysterectomy for uterine myoma from *menstruant nonpregnant human females*.

Fig. 1 shows few fluorescent fibers in the fimbrial and ampullary parts of the muscular wall (Fig. 1 A) and these appear almost exclusively around blood vessels. This is in contrast to the abundant fluorescence in the isthmic part of the wall (*Fig. 1 B*). The rich fluorescence *is here selectively localized to the inner circular muscle layer* which gradually increases in size towards the uterus. Within the uterus the pronounced fluorescence of the intramural part (*Fig. 1 D*) is more noticeable since the adjacent myometrium is devoid of adrenergic nerve terminals except for the perivascular areas (cf. Falck 1964). Recently it has been histochemically demonstrated (Norberg 1964 a, b) that the smooth musculature of the digestive and urinary tracts are lacking direct adrenergic innervation except for certain sphincteric regions. Parallel estimations of the noradrenaline content in the human oviduct by a fluorimetric method (*Euler and Lishajko 1961*) showed approximately equal values (0.1—0.3 μg/g) in the isthmic, the ampullary and the infundibular thirds of the organ. The similarity of the noradrenaline content in the different parts, in spite of the histochemical results, could partly be due to the presence of a plexus of adrenergically innervated vessels along the ampullary and infundibular parts. This plexus could not be found in the isthmic region on our slides.

Thus it appears possible that the symptom of isthmic spasm may be due to irregularities in the function of the sympathetic nerves supplying the isthmus. It is also evident that such irregularities could influence fertility by affecting ovum transfer and sperm migration through the Fallopian tube.

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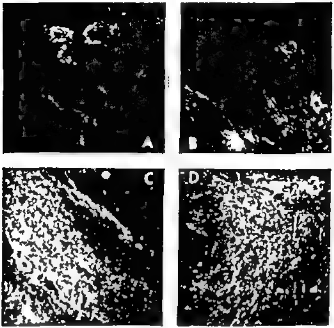

Fig 1 A Ampullary wall Slightly tangential section Single nerve terminals in musculature and around blood vessels 000 ×
B Isthmic wall 2 cm from uterus Oblique section Numerous terminals in inner circular layer 000 ×
C External tubo-uterine junction Longitudinal section Intense fluorescence from nerve terminals in inner circular layer Lumen upper right 000 ×
D Intramural part Cross section Abundant innervation of inner circular layer 000 ×

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